

LncRNA DCST1-AS1 accelerates the proliferation, metastasis and autophagy of hepatocellular carcinoma cell by AKT/mTOR signaling pathways

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Abstract. – **OBJECTIVE:** The previous research revealed that long noncoding RNAs (lncRNAs) play a vital role in the development of hepatocellular carcinoma (HCC). To further discuss the underlying mechanisms of lncRNA DCST1-AS1 in the pathogenesis of HCC.

PATIENTS AND METHODS: We screened the abnormally expressed genes in HCC tissues through microarray analysis and found that lncRNA DCST1-AS1 was one of the genes significantly up-regulated. Real Time-Polymerase Chain Reaction (RT-qPCR) was used to test the gene expression of lncRNA DCST1-AS1 in HCC tissues and HepG2 cells. Respectively, CCK-8 assay, flow cytometry detection, transwell assay, wound healing assay, transmission electron microscopy, and immunofluorescence staining were used to assess the proliferation, apoptosis, migration, and autophagy of HepG2 cells. Meanwhile, the expression of signaling pathway proteins was detected by Western blot.

RESULTS: lncRNA DCST1-AS1 was confirmed hyper-expression both in HCC tissues and HCC cells. High expression of lncRNA DCST1-AS1 was significantly correlated with inferior prognosis. Moreover, lncRNA DCST1-AS1 depletion suppressed proliferation and accelerated apoptosis, activated cycle arrest, restrained cell migration, and stimulated autophagy in HCC cells. In addition, it is found that the depletion of lncRNA DCST1-AS1 on HepG2 cells exhibits anti-tumor characteristics and was mediated by the AKT/mTOR signal transduction pathway. Furthermore, pre-treated HepG2 cells with SC79, an AKT signal activator, partially restored the effect of lncRNA DCST1-AS1 silencing on HepG2 cell proliferation, apoptosis, migration, and autophagy.

CONCLUSIONS: Our results suggested that lncRNA DCST1-AS1, as a carcinogenic factor in HCC, promoted cell proliferation, and invasion, inhibited apoptosis and autophagy by modulating

the AKT/mTOR signaling cascade. Therefore, our findings showed that lncRNA DCST1-AS1 may improve potential treatment strategies for HCC.

Key Words:

lncRNA DCST1-AS1, hepatocellular carcinoma cell, AKT, mTOR.

Abbreviations

HCC, Hepatocellular carcinoma; lncRNAs, Long non-coding RNAs; OS, Overall survival; siRNA, small interfering RNA.

Introduction

Hepatocellular carcinoma (HCC) is the sixth-largest malignancy with increasing incidence worldwide and results in more than half a million deaths worldwide per year¹. Despite advances in diagnostic and therapeutic techniques, the therapeutic efficacy of advanced stage HCC is still poor². Thus, it is crucial to reveal the pathogenic mechanisms underlying HCC and discover the novel targets for HCC treatment. Long non-coding RNA (lncRNAs) refers to non-protein coding RNA (ncRNAs) with a length of more than 200 nucleotides³. Although lacking the ability of coding protein, lncRNAs have recently been found to play a potent biological role in carcinogenesis in different types of cancer by regulating gene expression⁴. In HCC, cumulative evidence has shown that lncRNAs positively modulates growth, apoptosis, migration, and autophagy of hepatoma cells⁵. For example, the down-regu-

lated expression of lncRNA OGFRP1 can suppress HCC progression through AKT/mTOR and Wnt/ β -catenin signaling pathways⁶. Also, a recent study⁷ demonstrated that lncRNA THOR can markedly promote the proliferation and metastasis of HCC cells. On the contrary, the expression of lncRNA HOTAIRM1 is abnormally down-regulated, which defended against the development of HCC by suppressing the Wnt signaling pathway⁸. However, there are many lncRNAs that have not yet been identified and the identification and functional annotation of lncRNAs is a very promising research field to explore the regulation mechanism of biological processes in HCC.

In this work, we hope to discuss the function of a new lncRNA DCST1-AS1, which has never been studied in HCC before. Using microarray analysis, lncRNA DCST1-AS1 was confirmed up-regulated and had a significant correlation with the prognosis of HCC. However, little is known about the biological function and role of lncRNA DCST1-AS1 in the progression of HCC. We studied the impact of depletion of lncRNA DCST1-AS1 on HepG2 cell functions, including HepG2 cell proliferation, apoptosis, cell cycle, migration, and autophagy. Furthermore, to explain the biological mechanisms of lncRNA DCST1-AS1, we analyzed the AKT/mTOR signaling pathway through small interfering RNA (siRNA) and pre-treated with an AKT signal activator. Our report showed that lncRNA DCST1-AS1 was up-regulated in HCC tissues and promoted the development of HCC through the AKT/mTOR pathway. Therefore, this research suggested that lncRNA DCST1-AS1 may improve potential treatment strategies for HCC.

Patients and Methods

Ethics Statement

This investigation was approved by the Hubei Institute for Nationalities Committee and the Affiliated Min da Hospital Ethics Committee, Min da Hospital.

Reagents

Dimethyl sulfoxide (DMSO; Shanghai, China), EdU, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Akt, P-Akt, Bax, mTOR, P-mTOR, Bcl-2 were purchased from Affinity Biosciences; Cincinnati, OH, USA); Caspase-3, Cyclin D1, Cyclin B1, Cdc2, β -catenin, N-cadherin, E-cadherin, P62, Beclin1, and LC3I/II an-

tibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS) were obtained from Gibco Life Technologies (Grand Island, NY, USA).

Patients and Specimens

We collected fresh HCC tissues and paired adjacent liver tissues from 80 HCC patients at the Affiliated Min da Hospital of Hubei Institute for Nationalities of Hubei Province from 2012 to 2013. Surgery as the initial treatment mode and complete data and the specimens were immediately frozen in liquid nitrogen, then stored in a -80°C . We considered the time interval between the date of surgery and the date of death or the last follow-up as the overall survival (OS).

Cell Lines and Culture

Human hepatocellular carcinoma cell line HepG2 was derived from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells cultured in DMEM medium adding 10% FBS, incubator temperature is 37°C with 5% of CO_2 concentrations.

Cells Transfection

Three specifically targeting lncRNA DCST1-AS1 were constructed and synthesized by Ribobio (Guangzhou, China). Cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Then, we selected the most stable silencing effect sequence to continue with the following experiment. The target sequences were as follow: si-DCST1-AS1: sense 5'-GAAAGUGACACAAGCAATT-3' and antisense 5'-UTTTUGUTGTGTUGTTAA-3', HepG2 si-DCST1-AS1 cells and the control cells were treated with 10 μM SC79 or left untreated, then subjected to Cell Counting Kit-8 (CCK8), flow cytometry detection, transwell (24 h) and immunofluorescence for autophagy.

RT-PCR

TRIzolTM reagents (Invitrogen, Carlsbad, CA, USA) were used to isolate HCC cells total RNA and the extracted mRNA was reversely transcribed to complementary Deoxyribose Nucleic Acid (cDNA) according to the instructions of RT-PCR system. The DCST1-AS1 and GAPDH primer sequences were as follows: DCST1-

AS1-forward: 5'-TTCGTCTGGTCCCAATGTGTGG-3', DCST1-AS1-reverse: 5'-AAGCAGGACGAGTAAACCAACC-3', GAPDH-forward: 5'-CGCTCTCTGCTCCTCCTGT-3', GAPDH-reverse: 5'-ATCCGTTGACTCCGACCTA-3'.

Cell Proliferation Assay

The CCK-8 assay was used to test cell proliferation. HepG2 cells were seeded in a 96-well plate, after every 24 h inoculation, si-DCST1-AS1 or si-Control were transfected. Next, cell cultured with 10 μ L CCK-8 solutions for 1 h in the dark. Microplate reader (Bio-Rad, Hercules, CA, USA) was utilized to access the absorbance (OD) values at 450 nm.

Western Blot Analysis

The bicinchoninic acid (BCA) method was used to detect protein concentration following the instructions. Protein samples of the same amount (40 g/lane) were isolated by 10-12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). 5% bovine serum albumin (BSA) was employed to block membranes 1 h at room temperature. Then, the membranes were probed with primary antibodies (Bax 1:1000, caspase-3 1:800, Bcl-2 1:800, CyclinB1 1:1000, Cdc2 1:1000, CyclinD1 1:2000, β -catenin 1:1000, N-cadherin 1:800, E-cadherin 1:1000, LC3I/II 1:800, mTOR 1:2000, and phosphorylated (p)-mTOR 1:800, Akt 1:1000, phosphorylated (p)-Akt 1:800, P62 1:1000, Beclin1:2000) overnight at 0-4°C in ice-box and subsequently with an appropriate secondary antibody (goat anti-rabbit IgG 1:3000, and goat anti-mouse immunoglobulin (Ig) G 1:4000) 1 h at 4°C in ice-box. Enhanced chemiluminescence (ECL) kits were used to visualize the protein bands and Image Quant LAS 4000 Mini system was used to capture images (GE Healthcare Life Sciences, Chalfont, UK). Image J software (NIH, Bethesda, MD, USA) was used for image processing and the density method was used for quantitative analysis.

Cell Invasion Assays

Cell invasion assays were verified by wound healing experiment. Cells were seeded in 6 well plates and then scratched with a 200 μ L pipette tip until 100% confluence. Following treatment of 0 and 24 h, photos were taken subsequently, images depicting the migration of cells on the scratches were taken in 5 randomly selected areas.

Flow Cytometry Detection

Annexin V-FITC/PI apoptosis kits (BioVision, Inc. Milpitas, CA, USA) were applied to detect apoptosis as per manufacturer's instructions. After cultured in 6-well plates for overnight, cells were harvested, centrifuged, washed and then incubated with Annexin V-FITC/PI following standard protocols. Lastly, the detection and analyzing of apoptosis using a Guava Easy Cytometer (Germany).

Transwell Assay

Transwell assays were used for cell invasion detection. Briefly, 2×10^5 cells were cultured in the upper chamber with serum-free DMEM medium of a Matrigel-coated Boyden chamber, contained a lower chamber with DMEM containing 20% FBS. After 24 h incubation, cells were fixed with 10% formalin for 4 h, and then dyed with crystal violet (Beyotime, Shanghai, China) for 5 min. The number of cells was then counted under an Olympus microscope.

Transmission Electron Microscopy

Microstructure changes of autophagosomes and autophagic vacuoles were detected by Transmission electron microscopy. The slides of cells counterstained with 0.3% lead citrate and then observed on a transmission electron microscope. Autophagic vacuoles are defined as cells with 5 or more autophagic vacuoles.

Microarray Analysis

The Human 8 $\times$ 60 k LncRNA Expression Microarray V 3.0 from Ribobio (Guangzhou, China) was applied to detect gene microarray hybridization. Total RNA was quantified using NanoDrop 2000. The cDNA was labeled according to the Nimblegen gene expression analysis protocol when total RNA was synthesized. Lastly, Nimble Scan (Nimblegen, Madison, WI, USA) was conducted for data analysis.

Immunofluorescence Staining

Cells were seeded into 6-well plates and cultured on coverslips for 24 h, and then pre-treated with different stimuli for 24 h. After rinsed three times with phosphate-buffered saline (PBS), coverslips were fixed with 4% paraformaldehyde for 30 min, and then permeabilized with 0.1% Triton X-100, blocked in 10% goat serum, and incubated with polyclonal antibodies overnight at 4°C. On the next day, coverslips were incu-

bated with secondary antibody and 4', 6-diamidino-2-phenylindole (DAPI) was added to stain the nuclei. Lastly, slides were detected under an Olympus fluorescence microscope. Results were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA) and EdU immunofluorescence staining was observed by the EdU kits.

Statistical Analysis

All statistics were analyzed using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Mean $\pm$ SD was used for counting dates and two-tailed *t*-test and Chi-square tests were used for statistical analysis. $p \leq 0.05$ was considered statistically significant.

Results

The Expression of LncRNA DCST1-AS1 was Up-Regulated in Human HCC Tissues and Closely Related to Poor Prognosis

The results of the microarray analysis showed that DCST1-AS1 expression was significantly up-regulated in HCC tissues (Figure 1A). We also verified that the expression level of DCST1-AS1 in HCC tissues was significantly higher than that in adjacent liver tissues by RT-PCR (Figure 1B). Furthermore, the expression level of DCST1-AS1 in HepG2 cell line was markedly higher than that of normal hepatocyte HL7702 (data not shown). Additionally, according to the median expression ratio, we divided patients into two groups: lower lncRNA DCST1-AS1 group and higher lncRNA

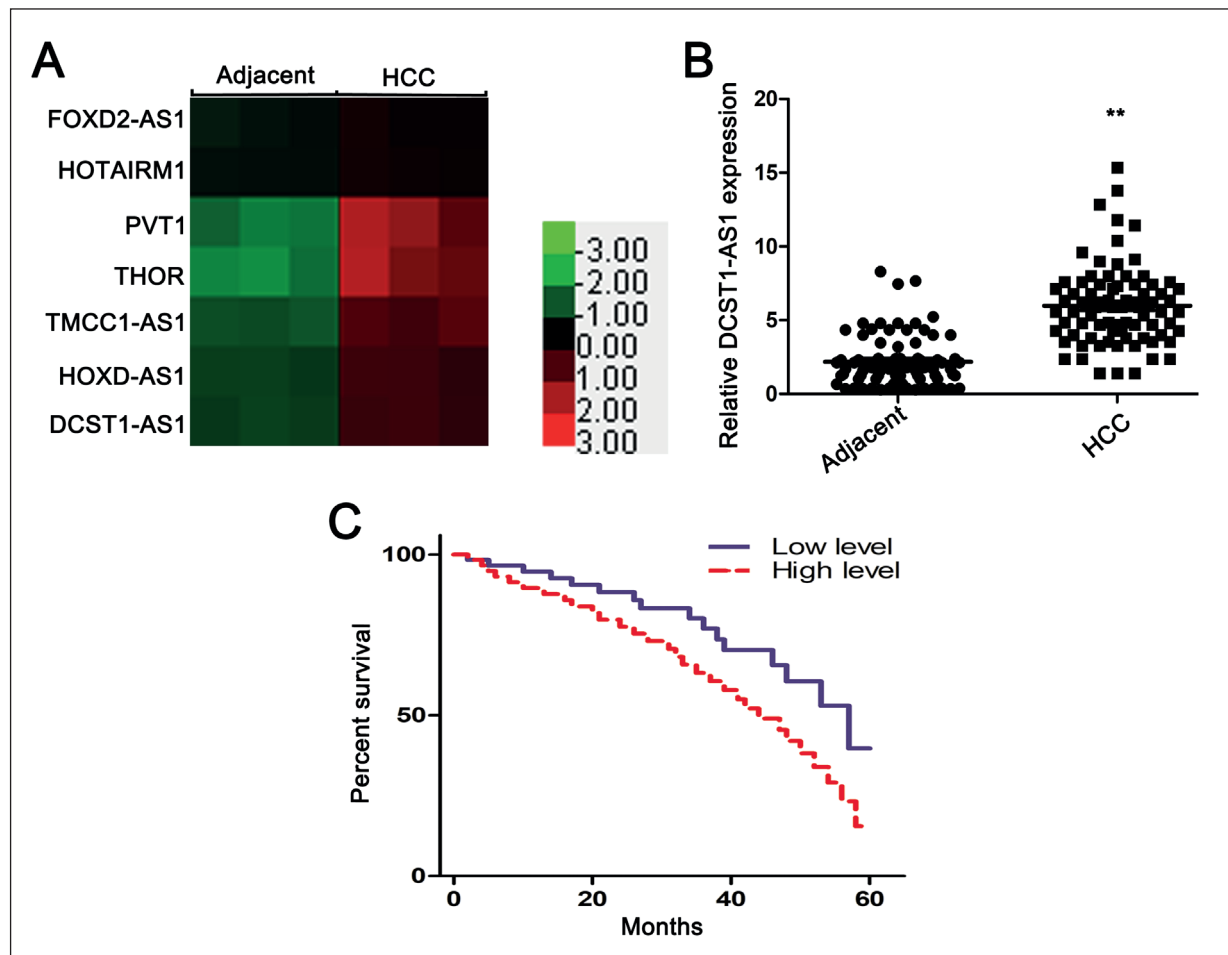


Figure 1. LncRNA DCST1-AS1 was up-regulated in human HCC tissues and associated with inferior prognosis. **A**, Heatmap indicated that lncRNA DCST1-AS1 was up-regulated in tumors. **B**, LncRNA DCST1-AS1 expression in 80 HCC tissue specimens and neighboring healthy liver tissues. **C**, OS curves were analyzed by lncRNA DCST1-AS1 levels. Patients with higher lncRNA DCST1-AS1 expression had a shorter survival time than those with lower expression ($p=0.023$). (* $p < 0.05$, ** $p < 0.01$ compared with control).

DCST1-AS1 group (DCST1-AS1 expression ratio $\leq$ median ratio, and DCST1-AS1 expression ratio $>$ median ratio). Next, we examined the survival analysis by Kaplan-Meier and log-rank test. As shown in Figure 1C, compared with higher lncRNA DCST1-AS1 group, the total survival time of the lower expression group in HCC tissues was longer ($p=0.023$). These results suggest that lncRNA DCST1-AS1 may play an important role in the development of HCC.

LncRNA DCST1-AS1 Depletion Inhibited Proliferation and Increased Apoptosis of HCC Cells

To detect whether the depletion of DCST1-AS1 can inhibit the proliferation of HCC cells, cells were evaluated by the EdU assay, which was used to detect the active synthesis of DNA in the cell cycle. As shown in Figure 2A, after transfection with si-DCST1-AS1, the cell proliferation rate was significantly suppressed. Also, CCK-8 assay showed that the proliferation of HepG2 cells was significantly inhibited after the silencing of lncRNA DCST1-AS1 (Figure 2B). Our results indicated that cell proliferation of HCC cells was significantly reduced after lncRNA DCST1-AS1 depletion. Furthermore, we investigated the potential apoptosis promoting mechanism after depletion of DCST1-AS1. Western blot was used to detect the important apoptosis-associated proteins: Bax, Bcl-2 and caspase3. Compared with control group, the depletion of lncRNA DCST1-AS1 significantly induced the expression level of Bax and significantly reduced the expression of Bcl-2 (Figure 2C-D). In addition, the treatment with si-DCST1-AS1 increased the expression of cleaved caspase3. To validate our results, using Annexin V-FITC/PI double staining, we also performed flow cytometry to detect the apoptosis effects of the lncRNA DCST1-AS1 knockdown. Our results showed that the percentage of apoptotic cells significantly increased after silencing lncRNA DCST1-AS1 (Figure 2E and F). These findings confirmed the positive role of lncRNA DCST1-AS1 in proliferation and apoptosis tolerance of HepG2 cells.

Downregulation of LncRNA DCST1-AS1 Induces Cell Cycle Arrest in HCC

Cell cycle acceleration is one of the mechanisms for the rapid progression of HCC and induction of the cell cycle arrest is an effective method to inhibit⁹. Therefore, flow cytometry was performed to study cell cycle when lncRNA

DCST1-AS1 depletion. As shown in Figure 3A and B, compared to si-control group, lncRNA DCST1-AS1-silencing HepG2 cells were significantly blocked at G1 phase. Furthermore, using Western blot analysis, we detected the expression of cyclin molecules, including cyclin B1, cyclin D1, and Cdc2, which are involved in the regulation of cell cycle. Compared to si-control group, our results indicated that lncRNA DCST1-AS1 depletion markedly inhibited the expression of cyclin D1, cyclin B1, and Cdc2. Our findings suggested that the knockdown of lncRNA DCST1-AS1 may be involved in G1-phase arrest in HepG2 cell cycle progression.

Silencing LncRNA DCST1-AS1 can Inhibit the Migration and Invasion of HCC Cells

Migration and invasion often promote tumor progression and metastasis, which is an important feature of malignant tumor cells¹⁰. Hence, we analyzed the effect of silencing lncRNA DCST1-AS1 on the migration and invasion of HepG2 cells through wound healing experiment and transwell assay. Results from statistical analyses of wound healing experiments yielded that the knockdown of lncRNA DCST1-AS1 prominently increased the migration speed of HepG2 cells (Figure 4A and B). Similarly, with the result of transwell invasion assay, as shown in Figure 4C and D, compared to the control cells, the mobility of transfected si-DCST1-AS1 cells was significantly lower. Next, we assessed whether si-DCST1-AS1 affects migration regulatory proteins, such as β -catenin, N-cadherin, and E-cadherin.

Notably, the knockdown of lncRNA DCST1-AS1 significantly inhibited the expression of β -catenin, N-cadherin. On the contrary, the expression of E-cadherin was increased (Figure 4E and F). All of our results indicated that the down-regulation of lncRNA DCST1-AS1 could inhibit HepG2 cell migration and invasion.

LncRNA DCST1-AS1 Knockdown Induced Autophagy in HCC Cells

Autophagy plays a vital role in many physiological processes such as cell development, proliferation, and apoptosis. It also plays a crucial role in the occurrence and development of malignant tumors¹¹. To test the promotion of autophagy by lncRNA DCST1-AS1 knockdown in HepG2 cells, we observed the morphological changes in DCST1-AS1 depletion cells by electron microscopy and LC3 immunofluorescence staining anal-

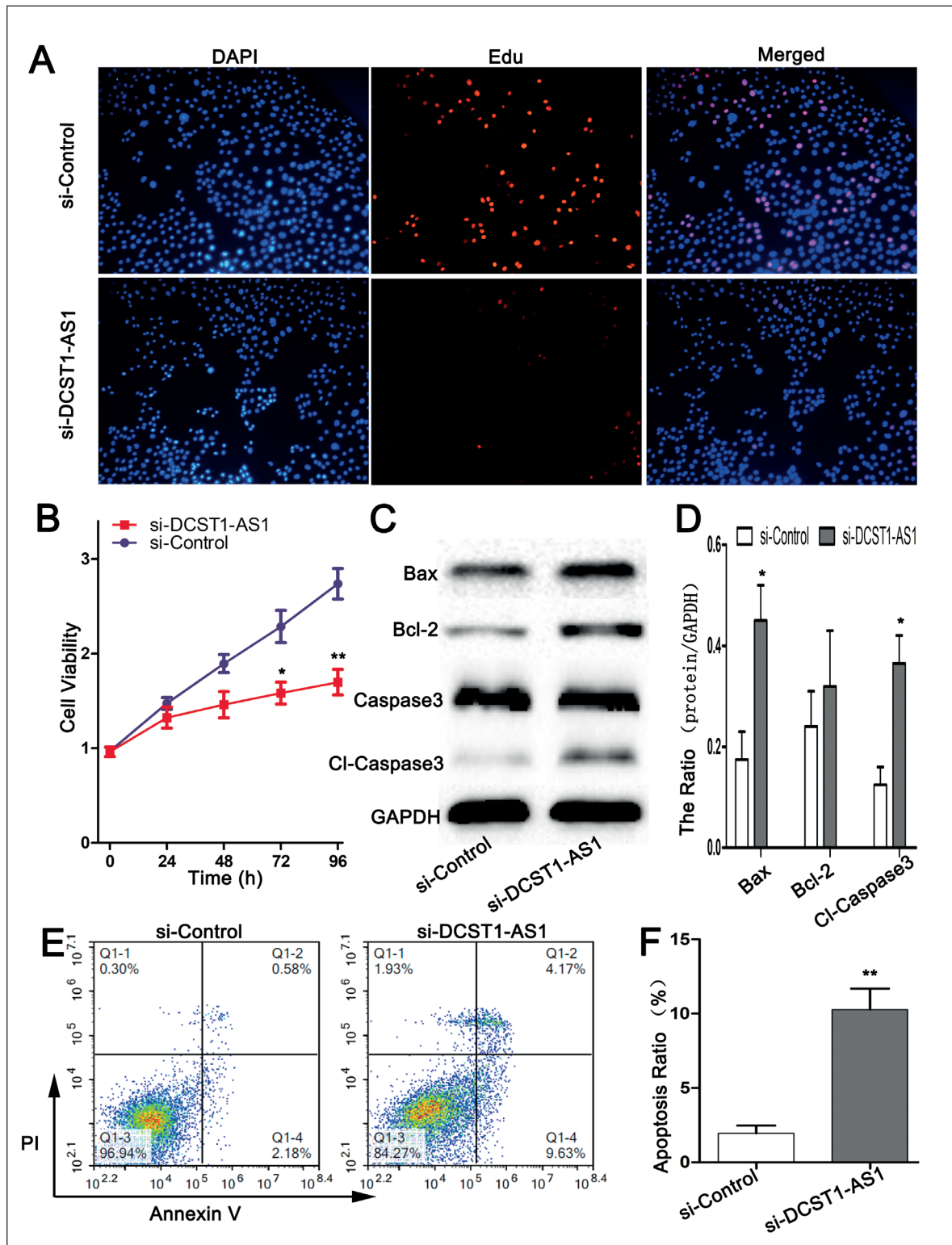


Figure 2. LncRNA DCST1-AS1 depletion inhibited proliferation and promoted apoptosis of HCC cells. **A**, EdU assays indicated that LncRNA DCST1-AS1 depletion inhibited cell proliferation compared with control exposure. **B**, Cell viability as illustrated via CCK8 assay. **C**, Expressions of Bax, Bcl-2, and caspase3 were analyzed with Western blotting and **D**, Quantitation data of **C**. **E**, Representative flow cytometry scatter plots of propidium iodide (PI) (Y axis) vs. Annexin V-fluorescein isothiocyanate (FITC) (X axis) and **F**, Quantitation data of average of 3 independent flow cytometry experiments. (* $p < 0.05$, ** $p < 0.01$ compared with control, $n=3$).

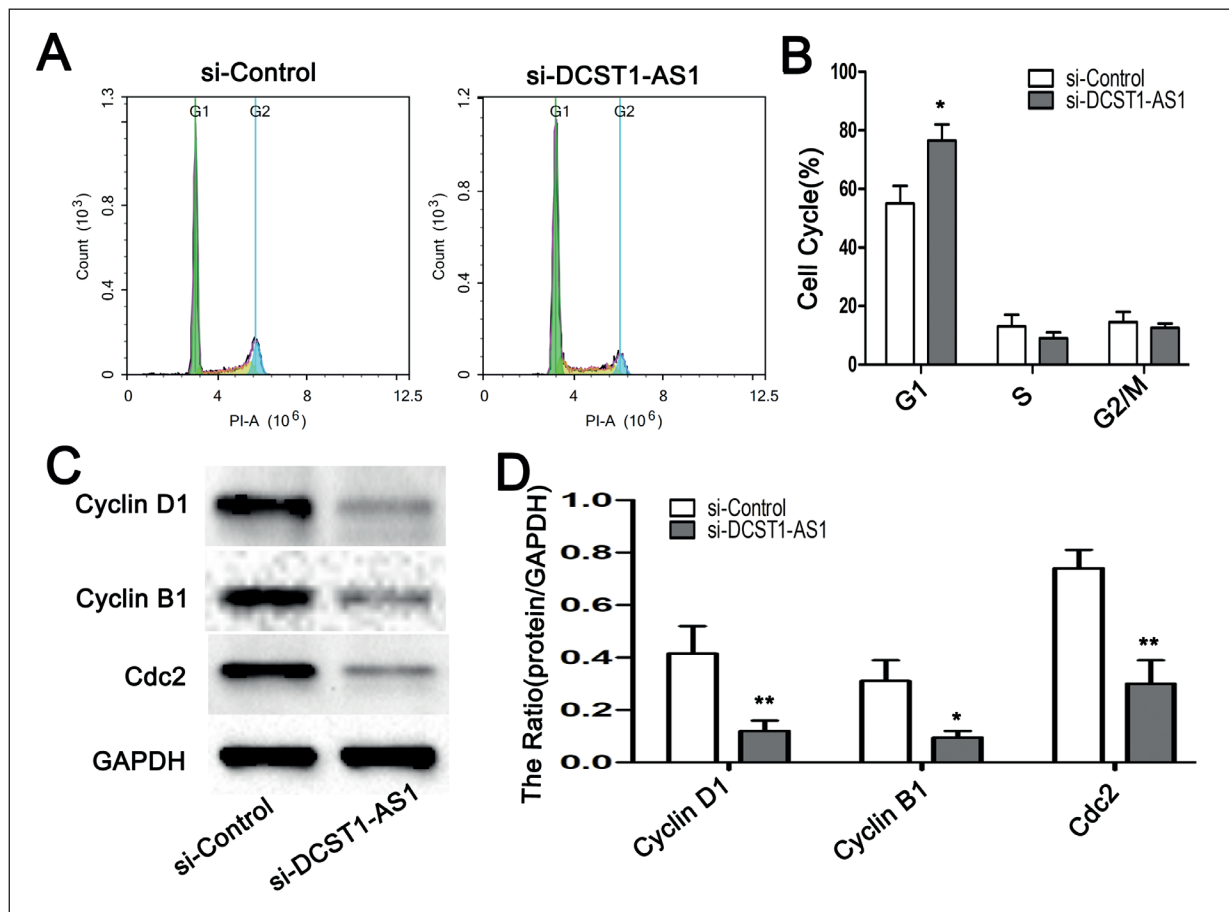


Figure 3. Induction of HCC cell cycle arrest by downregulation of lncRNA DCST1-AS1. **A**, Representative flow cytometry cell cycle of PI, and **B**, Quantitation data of flow cytometry cell cycle of 3 independent experiments. **C**, Cell cycle proteins expression of Cyclin D1, Cyclin B1, and Cdc2 by Western blot analysis. **D**, Quantitation data of **C**, (* $p < 0.05$, ** $p < 0.01$ compared with control, $n=3$).

ysis. As shown in Figure 5A and B, compared with control group, more autophagic vesicles, and autophagosomes with double-membrane vacuolar structure appeared in the si- DCST1-AS1 depletion treated HepG2 cells. Similarly, DCST1-AS1 silencing induced LC3 fluorescence vesicles increased significantly compare to si-control cells (Figure 5C and D). Next, we detected the expression of Beclin1, LC3, p62, and other autophagy-related genes by Western blot. We found that DCST1-AS1 depletion up-regulated the expression of Beclin1 and increased expression of p62. Also, LC3-II accumulation was more pronounced in DCST1-AS1 knockdown HepG2 cells than si-control group (Figures 5E, 5F, and G). Our results indicated that lncRNA DCST1-AS1 were closely related to the autophagosome formation and the regulation of autophagy-related genes of HepG2 cells.

LncRNA DCST1-AS1 Promoted the Progression of HCC Cells Through the AKT/Mtor Signaling Pathway

It has been reported that AKT/mTOR cascade plays a vital role in regulating HCC cell proliferation, apoptosis, cell cycle, invasion and autophagy¹². To determine the relation between lncRNA DCST1-AS1 and AKT/mTOR signaling pathway, we down-regulated lncRNA DCST1-AS1 in HepG2 cells and observed the expression and activity changes of AKT and mTOR proteins.

Activation of AKT and mTOR is dependent on protein phosphorylation. As shown in Figures 6A, B, and C, silenced lncRNA DCST1-AS1, both AKT and mTOR phosphorylation levels in HepG2 cells were significantly down-regulated. Our results demonstrated that lncRNA DCST1-

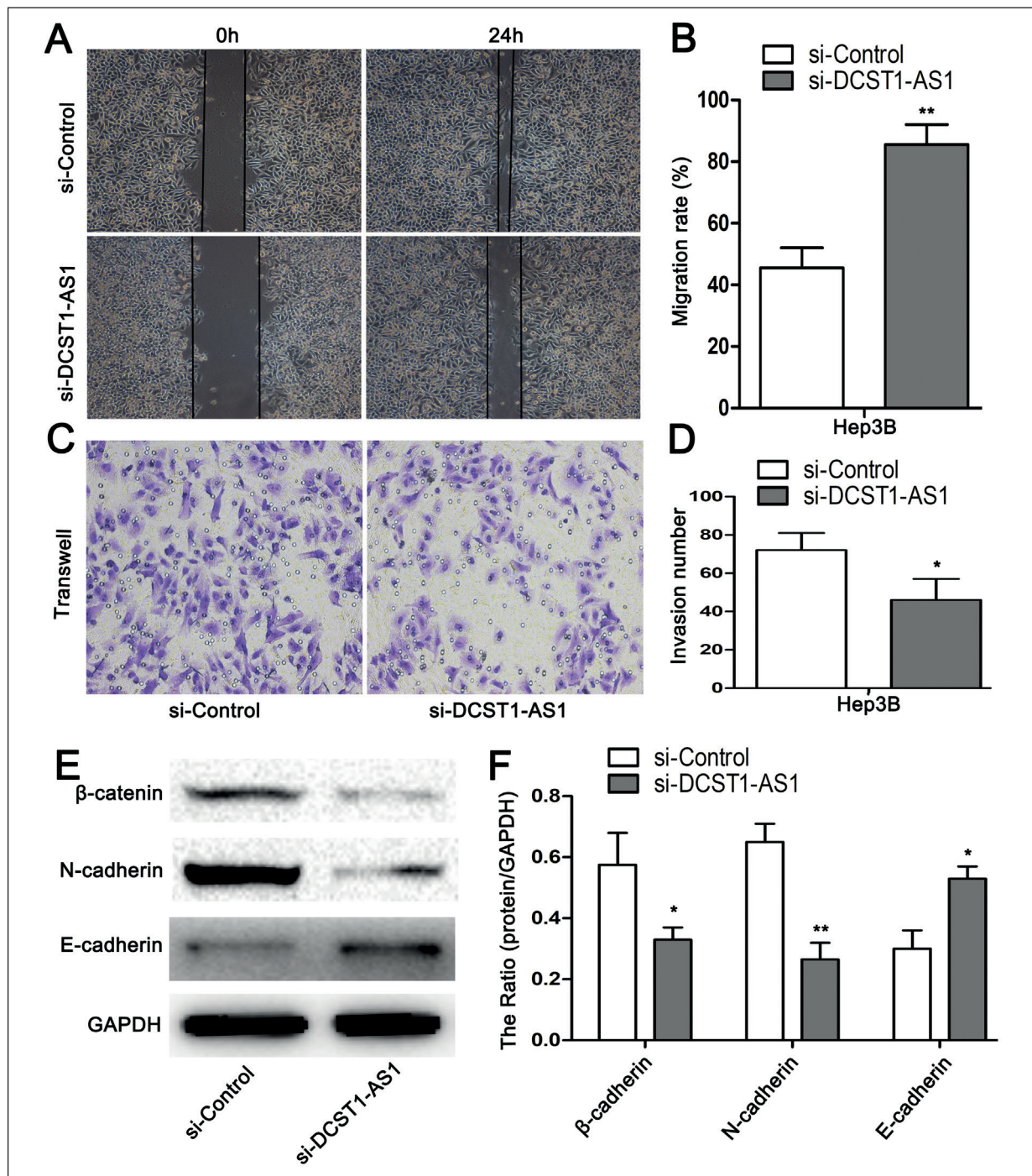


Figure 4. Knockdown of lncRNA DCST1-AS1 inhibited HCC cell migration and invasion. **A**, Monolayer of cells was scraped with a pipette tip and then treated with si - lncRNA DCST1-AS1. The migrating cells were assessed with a microscope equipped with a camera (200×), and **B**, Quantitation data of **A**, **C**, Invasive ability was evaluated by a transwell invasion assay (200 ×), and **D** Migratory cells in the bottom surface of the membrane were counted. **E**, Full-length blots of β-catenin, N-cadherin, E-cadherin, and GAPDH are presented. **F**, Quantitation data of **E**, (* $p < 0.05$, ** $p < 0.01$ compared with control, $n=3$).

AS1 was closely related with activated of AKT/mTOR signaling pathway in HepG2 cells. Moreover, AKT activator SC79 was applied to evalu-

ate the role of lncRNA DCST1-AS1 down-regulation in the cell changes of the AKT signaling pathway. Of note, activation of AKT can partial-

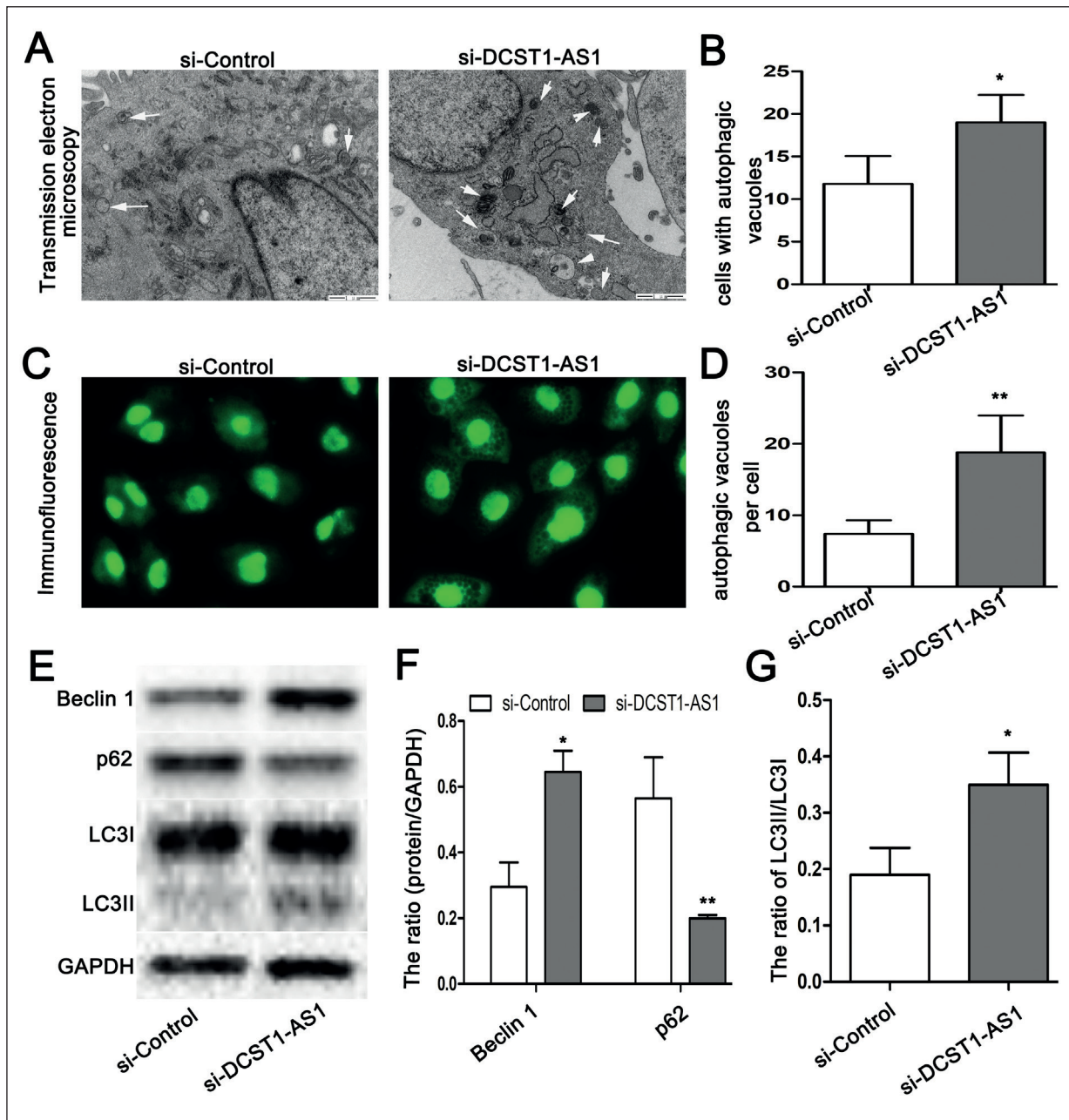


Figure 5. Silencing of lncRNA DCST1-AS1 induced autophagy in HCC cells. **A**, Representative transmission electron microscopy images of HepG2 cells treated with si-lncRNA DCST1-AS1 or si-control. **B**, Bar chart showing quantitation data of the cells with autophagic vacuoles which were defined as cells that had five or more autophagic vacuoles. **C**, LC3 immunofluorescence staining of HepG2 cells treated with si-lncRNA DCST1-AS1 or si-control, and **D**, bar chart showing Quantitation data of average of 3 independent LC3 immunofluorescence staining. **E**, Expressions of Beclin 1, P62 and LC3 II/I were analyzed with Western blotting and **F**, **G**, Proteins expression were evaluated. (* $p < 0.05$, ** $p < 0.01$ compared with control, $n=3$).

ly reverse the restraint effect of lncRNA DCST1-AS1 silencing-mediated cell changes (Figures 6D, E, and F), which indicated that lncRNA DCST1-AS1 promoted the occurrence and development of HCC cells through the AKT/mTOR signaling pathway.

Activation of AKT by SC79 Partially Restored the Biological Changes in Hepg2 Cells Caused by Down-Regulation of LncRNA DCST1-AS1

To evaluate the ability of lncRNA DCST1-AS1 knockdown to target the AKT/mTOR cascade, we

tested whether SC79 could reverse the effect of DCST1-AS1 depletion on proliferation, apoptosis, migration, and autophagy of HepG2 cells. We used CCK8 assay to investigate the effect of SC79 on the proliferation of DCST1-AS1 knockout cells. As shown in Figure 7A, our results showed that pretreatment with SC79, the proliferation level of DCST1-AS1 depletion cells were increased to a similar degree of si-control cells. Moreover, the induction effects on HepG2 cells apoptosis of DCST1-AS1 depletion were partially antagonized when pretreatment with SC79 (Figures 7B and C). Those effects were similarly detected in the transwell assay (Figures 7D and E). Furthermore,

SC79 dramatically reduced the autophagosomes in LncRNA DCST1-AS1 knockdown cells (Figures 7F and G). All of these data clearly confirmed that the silencing of DCST1-AS1 inhibits HCC progression partly *via* AKT/mTOR cascade.

Discussion

Due to the lack of early diagnosis and poor prognosis, most of the patients are already at an advanced stage when HCC was first diagnosed¹³. Currently, there are no effective treatments for HCC, the 5-year survival rates are very low.

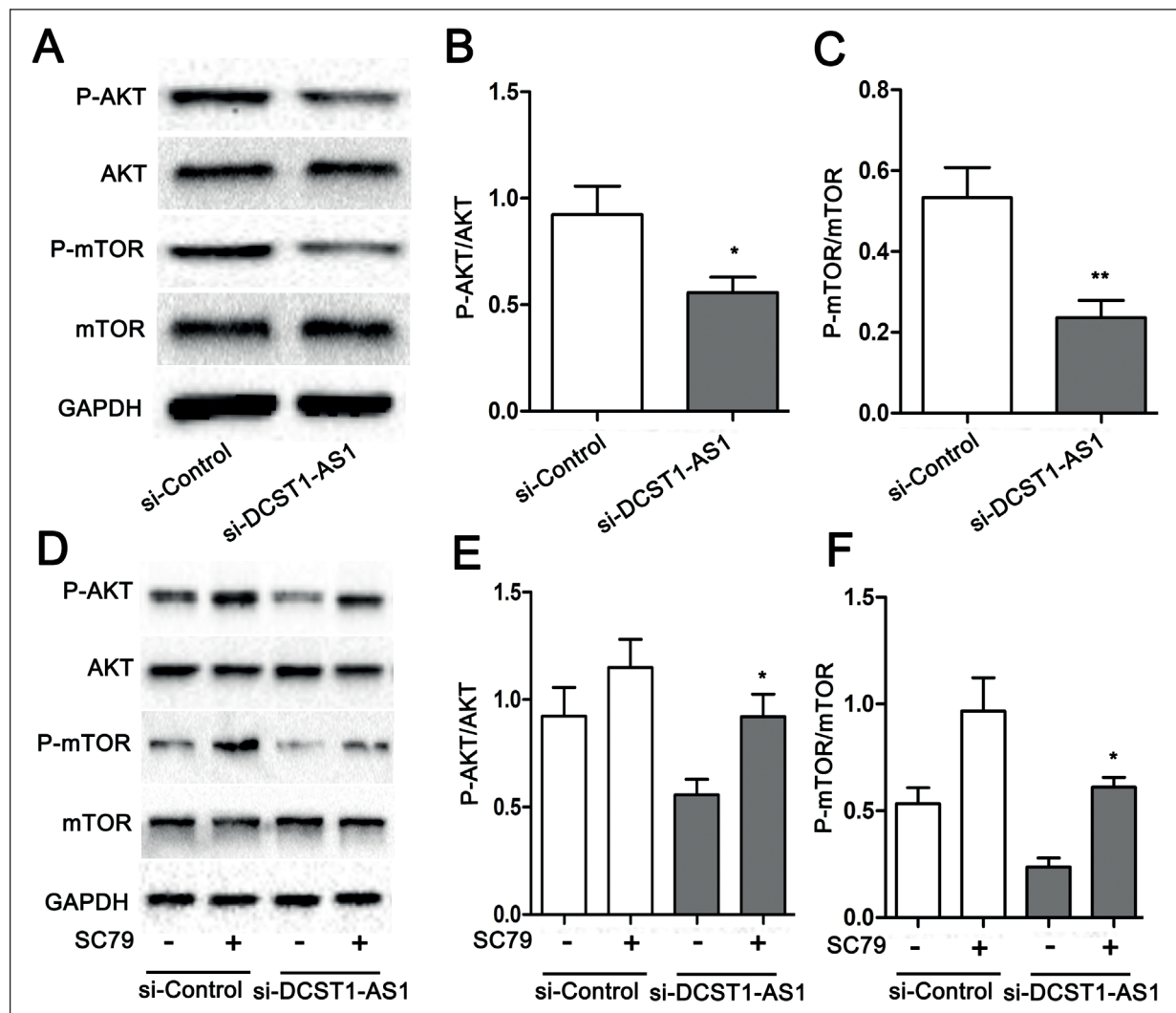


Figure 6. Effects of LncRNA DCST1-AS1 depletion on AKT/mTOR pathway signaling in HepG2 cells. **A**, the expression levels of p-AKT/AKT and p-mTOR/mTOR were measured by Western blotting. **B** and **C**, Quantitation data of **A-D**, Effects of SC79 and si-LncRNA DCST1-AS1 on the expression of p-AKT/AKT and p-mTOR/mTOR in HepG2 cells. **E**, and **F**, Quantitation data of **D**. (* $p < 0.05$, ** $p < 0.01$ compared with control, $n=4$).

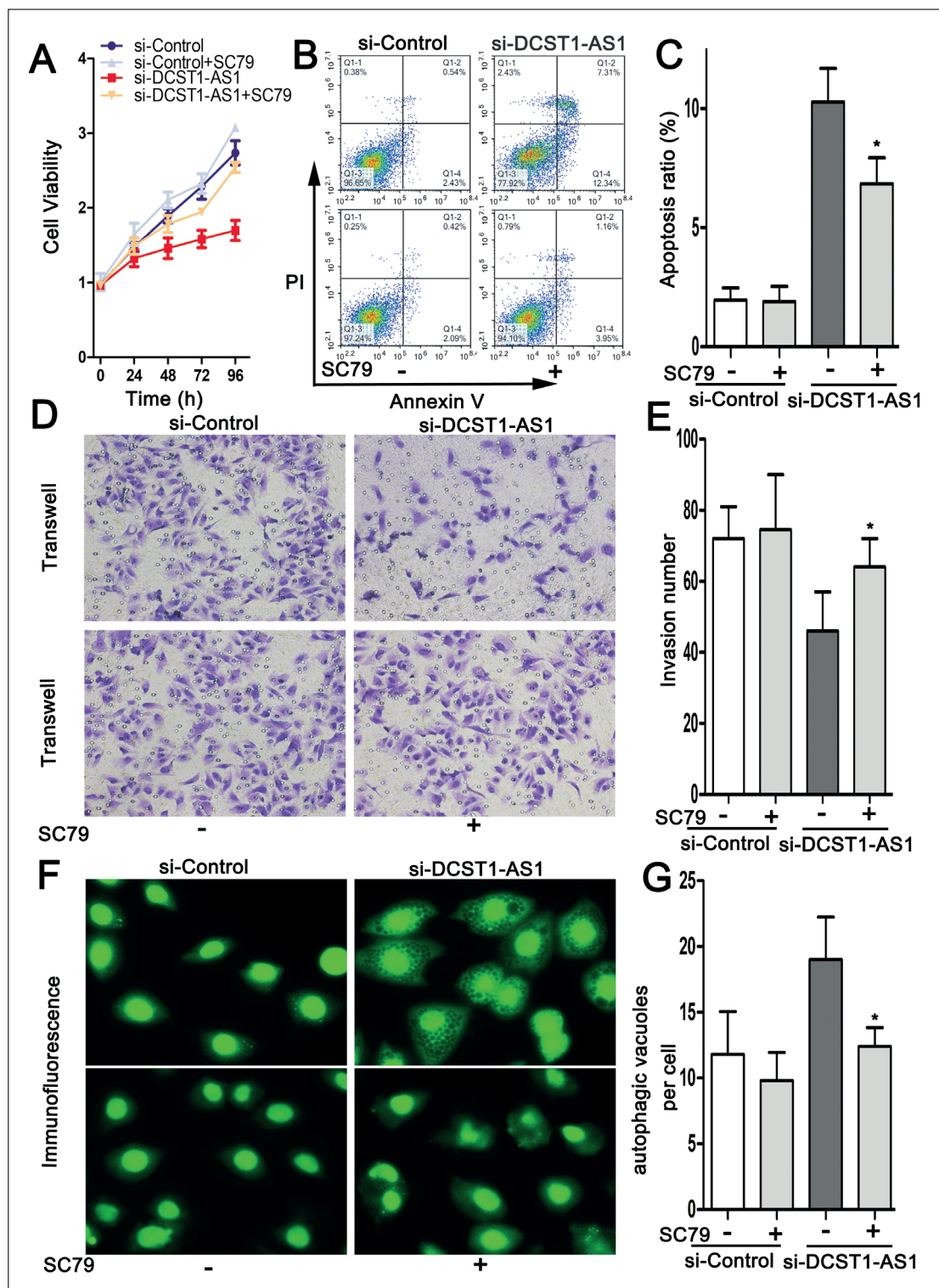


Figure 7. AKT activation by SC79 partially restores LncRNA DCST1-AS1 knockdown HepG2 cells. **A**, HepG2 cells were cultured with or without SC79 for indicated time and then cell proliferation was measured using MTT assay. **B**, Representative flow cytometry scatter plots of propidium iodide (PI) (Y axis) vs. Annexin V-fluorescein isothiocyanate (FITC) (X axis) and **C**, Quantitation data of average of 3 independent flow cytometry experiments. **D**, Migrating cells were assessed with a microscope equipped with a camera (200 $\times$), and **E**, Quantitation data of **E**, **F**, LC3 immunofluorescence staining of HepG2 cells treated with si-LncRNA DCST1-AS1 or si-control and cultured with or without SC79, and **G**, bar chart showing Quantitation data of average of 3 independent LC3 immunofluorescence staining. (* $p < 0.05$, ** $p < 0.01$ compared with control, $n=3$).

Thus, HCC has become the most common cause of malignancy and death in the worldwide¹⁴. The mechanisms of HCC initiation and progression remain unclear. Therefore, it is urged to explore the pathogenesis and provide the new effective treatment of HCC.

Emerging evidence of the human transcriptome has indicated that many lncRNAs may be critical to the development and progression of HCC¹⁵. In addition, although lncRNAs do not have the ability to encode proteins, through the regulation of signal transduction activation and target gene expression, they can regulate and participate in many HCC-related biological processes, such as cell proliferation, apoptosis, cell cycle, invasion, and autophagy¹⁶. Therefore, it is critical to elucidate the relevant mechanisms of lncRNA in the occurrence and development of HCC.

In this study, as well as in HepG2 cells, we showed that the expression of lncRNA DCST1-AS1 in HCC tissues was significantly higher than that in adjacent non-tumor tissues. The higher lncRNA DCST1-AS1 expression, the poorer prognosis of HCC patients. Moreover, silencing of lncRNA DCST1-AS1 markedly decreased HepG2 cells proliferation and accelerated apoptosis. In addition, compared to control groups, the similar results were found in HepG2 cell migration, invasion, and autophagy when treated in the silencing of lncRNA DCST1-AS1. Interestingly, we last found that those biological effects of the silencing of DCST1-AS1 inhibit HCC progression partly *via* AKT/mTOR cascade.

HCC cell growth is complex and pernicious processes which were regulated by multiple factors including cell proliferation, apoptosis-resistant¹⁷. He et al¹⁸ revealed that Bax is executed as a procedure for apoptosis, while Bcl-2 inhibits Bax activation to inhibit apoptosis, and the ratio of Bax/Bcl-2 is a good indicator for apoptosis assessment. We observed the expression of Bax and Bcl-2 proteins were significantly changed in the silencing of lncRNA DCST1-AS1 compared with control groups by WB, both of which are recognized as driving forces of apoptosis. These results of our study demonstrated that lncRNA DCST1-AS1 can regulate the expression and the ratio of Bax and Bcl-2, thereby regulating proliferation and apoptosis.

Cell cycle also plays a potent role in regulating cell proliferation, and emerging evidence proved that cell cycle arresting is an effective way to restrain the proliferation of HCC cells¹⁹. It was clear

that cell cycle progression was regulated by various cyclin-related proteins, such as cyclin D1, cyclin B1, and Cdc2. Cyclin D1 is an activator of the G1/S transition, also, Cyclin B1 rapidly accumulates in S phase and then rapidly degrades during mid/late transformation. In addition, it has been already detected that cdc2 is involved in M-phase-inductive activities²⁰. Our results revealed that treatment of si-lncRNA DCST1-AS1 of HepG2 cells was induced to accumulate in the G1 phase of the cell cycle. Moreover, si-lncRNA DCST1-AS1-treated HepG2 cells significantly decreased the expression levels of Cyclin D1, Cyclin B1, and cdc2. We can conclude, by affecting the expression of cyclin D1, cyclin B1, and cdc2, lncRNA DCST1-AS1 could regulate HepG2 cell cycle progression, and thus promote HCC cells proliferation.

Although HCC metastasis consists of a series of consecutive steps, most deaths are caused by migration. Thus, interruption one or more of HCC cell migration, including cell adhesion and cell invasion have to be successfully studied²¹. In this study, we have found lncRNA DCST1-AS1 closely related to the migration and invasion of HCC cells. β -catenin, N-cadherin, as well as E-cadherin were reported to regulate cells migration in tumor metastasis. When knockdown lncRNA DCST1-AS1, the ability of cell migration was decreased and the expression of migration-related regulatory proteins was down-regulated. However, our results indicated that knockdown of lncRNA DCST1-AS1 promoted E-cadherin expression, suggesting that lncRNA DCST1-AS1 plays a vital in the regulation of HCC metastasis.

Recent studies have found a strong implicated between autophagy and the progression of several types of cancers. Huang et al²² demonstrated that autophagy acts conspicuously as a pro-apoptosis activator and increase autophagy may inhibit HCC progression. There is already growing evidence that anticancer drugs restrain tumors progression by stimulating the cellular autophagic process. This research demonstrated a prominent augment of the autophagic vacuole in HepG2 cells as well as increasing autophagy-related regulating marks in response to si-lncRNA DCST1-AS1 treatment, which describe the accounts as remarkably consistent with previous reports. This study supports this view that downregulation of lncRNA DCST1-AS1 expression may inhibit HCC progression though accelerating the process of autophagy.

Recently, it was demonstrated that AKT/mTOR signal pathway play important roles in HCC initiation and progression, including regulating cell proliferation, apoptosis, cell cycle, invasion, and autophagy²³. To determine whether lncRNA DCST1-AS1 plays a carcinogenic role in HCC by targeting the AKT/mTOR pathway, we identified the effects of lncRNA DCST1-AS1 on the AKT/mTOR cascade of HepG2 cells. In the present study, si-lncRNA DCST1-AS1 treatment significantly decreased the proportion of the activated forms of AKT and mTOR. However, the AKT activator SC79, which partially reversed the effects of lncRNA DCST1-AS1 silencing. In addition, to confirm its potential mechanisms, we detected whether the silencing of lncRNA DCST1-AS1 exerts anti-proliferation, pro-apoptosis, migration inhibition, and autophagy activity by regulating the AKT/mTOR cascade. Consistent with the WB results, intriguingly, SC79 partially alleviated the effects of lncRNA DCST1-AS1 silencing on proliferation, apoptosis, migration, and autophagy. The effects of lncRNA DCST1-AS1 on HCC progression is most likely due to its direct effect on AKT/mTOR pathway targets.

Conclusions

We supported that lncRNA DCST1-AS1 was significantly increased in HCC tissues and closely related to poor prognosis HCC patients. Moreover, knockdown lncRNA DCST1-AS1 suppressed cell proliferation, invasion, and promoted apoptosis and autophagy *via* regulating the AKT/mTOR cascade *in vitro*. Thus, our study demonstrates that lncRNA DCST1-AS1 may become a potential validity drug target that could be used for the therapeutic method of clinical HCC patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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