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# LncRNA DANCR promotes ATG7 expression to accelerate hepatocellular carcinoma cell proliferation and autophagy by sponging miR-222-3p

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**Abstract. – OBJECTIVE: LncRNA differentiation antagonizing non-protein coding RNA (DANCR) is an oncogene in various malignant cancers, including hepatocellular carcinoma (HCC). Autophagy is an intracellular self-digestion mechanism that accelerates the progression of HCC via promoting cell survival. However, the role of lncRNA DANCR in HCC, and the mechanism of lncRNA DANCR in the regulation of autophagy in HCC remains unknown. Therefore, the aims of this study are the investigation of the role of lncRNA DANCR in HCC, and the exploration of the molecular mechanism of lncRNA DANCR in regulating autophagy of HCC cells.**

**PATIENTS AND METHODS: In this study, the expression of lncRNA DANCR, miR-222-3p, and autophagy-related gene 7 (ATG7) was detected by qRT-PCR. The cell proliferation and colony formation were measured by Cell Counting Kit-8 (CCK-8) assay and colony formation assay. And the autophagic flux was evaluated by mRFP-GFP-LC3B reporter. The autophagy related proteins were analyzed by Western blotting. Besides, the relationship between lncRNA DANCR and miR-222-3p, as well as between miR-222-3p and ATG7, was determined by Dual-Luciferase reporter system.**

**RESULTS: We found high expression of lncRNA DANCR and ATG7, and low expression of miR-222-3p in HCC tissues and cell lines. And lncRNA DANCR positively correlated with poor survival of HCC patients. Moreover, the knockdown of lncRNA DANCR inhibited cell proliferation and autophagy of HCC cells. And we predicted and proved that lncRNA DANCR induced cell proliferation, colony formation and autophagy by increasing ATG7 and suppressing miR-222-3p.**

**CONCLUSIONS: Our study demonstrates the promoting role of lncRNA DANCR in HCC, and indicates the regulatory effects of lncRNA DANCR on regulating autophagy of HCC.**

*Key Words:*
LncRNA DANCR, MiR-222-3p, ATG7, Autophagy, HCC.

## Introduction

Liver cancer is the sixth malignant cancer, and mostly causes tumor-related mortality in the world[1]. Its incidence and mortality rates in men are higher than women. Hepatocellular carcinoma (HCC) is one of the primary liver cancers with 75%-85% incidence rates in all liver cancer cases[1]. The major risk factors of HCC include hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, aflatoxin contaminated-food, excessive alcohol intake, smoking etc.[2,3]. Although the development of treatment in HCC, there are still high diagnostic rates and mortality. Therefore, investigating the underlying molecular mechanism of aggressive progression in HCC is necessary for discover novel diagnostic and therapeutic strategies.

Autophagy is an important cell death pattern which maintains homeostasis by dissolving the damaged-organelles and macromolecules by lysosome[4]. It acts as a major role in malignant procession of multiple tumors[5,6], and exhibits dual regulatory effects on tumorigenesis[7,8]. In some cases, induction of autophagy accelerates neoplastic cell death to inhibit tumorigenesis[8,9]; oppositely, the promotion of autophagy facilitates neoplastic cell survival and proliferation[10,11]. In other cases, induction of autophagy promotes neoplastic cells resistance to the stimulation of hypoxia[12], metabolites[13] and therapeutic drugs[14,15].



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Thus, to explore the functions and regulatory mechanisms of autophagy is pivotal to investigate HCC pathogenesis.

Long non-coding RNAs (lncRNAs) are a group of non-coding RNAs with more than 200 nucleotides lengths, which have been more concerned in the field of cancer research. LncRNAs play a critical regulatory role in the development of HCC by modulating cell survival[16], proliferation[17], metastasis[18], invasion[17], and cell death, including apoptosis[19], pyroptosis[20], ferroptosis[19], autophagy[21], and other cell death patterns. LncRNA DNACR has been reported acting as an oncogene to obtain stemness features of HCC[22], facilitating cell growth, proliferation and metastasis by sponging miR-216a-5p[23] or miR-27a-3p[24]. Pan et al[25] have illustrated that lncRNA DNACR induces autophagy in osteosarcoma to promote osteosarcoma development. However, the mechanism of lncRNA DNACR modulating autophagy in HCC remains unknown.

In the present study, we demonstrated a high expression of lncRNA DNACR in HCC tissues and cell lines. The silence of lncRNA DNACR inhibited cell proliferation, colony formation and autophagy by upregulating ATG7 as a ceRNA *via* sponging miR-222-3p. These results provided the potential diagnostic biomarker and novel therapeutic strategy of HCC.

## Patients and Methods

### Specimen

A total of 62 paired HCC clinical specimens and normal specimens were provided by Qingdao University (Qingdao, China). All patients were confirmed according to the pathological examination and all surgeries were consented by all patients who were enrolled in this study. In this study, HCC patients were recruited with therapeutic hepatectomy, and HCC patients were excluded with neoadjuvant therapy or palliative hepatic resection. This study was approved by the Ethical Committee of Qingdao University (Qingdao, China).

### Cell Lines and Cell Culture

The human HCC cell lines (Bel7407, Hep3B, HepG2, Huh7 and MHCC97H) and the hepatocyte cell line LO2 were obtained from ATCC (Manassas, VA, USA). All cells were maintained in the complete medium including DMEM medium (Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum (FBS, HyClone, South Logan, UT, USA) and 1% penicillin/streptomycin solution (Sigma-Aldrich, St. Louis, MO, USA) in a humid incubator with 5% $CO_2$ at 37°C.

### qRT-PCR

Total RNA was extracted from specimens and cells using TRIzol reagent (TaKaRa, Dalian, China), and total RNA was reversed transcription into cDNA using PrimeScript™ 1st Strand DNA Synthesis Kit (TaKaRa, Dalian, China). Then, the cDNA was amplified using TB Green® Fast qPCR Mix (TaKaRa, Dalian, China). The primers in this study were as follows: DNACR, forwards, 5'-GCCCCCTGCCATCCATATAC-3', and reverse, 5'-AGTCACAGGCCTTTACACCG-3'; miR-222-3p, forwards, 5'-ACCCTCAATGGCTCAGTAGC-3', and reverse, 5'-GCCATCAGAGACCCAGTAGC-3'; ATG7, forwards, 5'-TGCACATTTCCTGTGGGGAT-3', and reverse, 5'-GGTGGGAGCACTCATGTCAA-3'; GAPDH, forwards, 5'-GCTCCCTCTTTCTTTGCAGC-3', and reverse, 5'-ACCATGAGTCCTTCCACGAT-3'; U6, forwards, 5'-CCCTTCGGGGACATCCGATA-3', and reverse, 5'-TTTGTGCGTGTCATCCTTGC-3'. The expression of genes was quantified by $2^{-\Delta\Delta Ct}$ methods, and normalized by GAPDH and U6.

### CCK-8 Assay

The HepG2 and Huh7 cell proliferation was performed by CCK-8 kit (Beyotime, Shanghai, China) referencing to the manufacturer's protocol. In brief, 2 x $10^4$ cells were plated into each well of 96-well plates and incubated for 24 h, 48 h, and 72 h after transfection. The cells of each well were stained with 10 μL CCK-8 reagent for 1 h, then, the absorbance of each well was measured at 450 nm in a microplate reader (BioRad, Hercules, CA, USA).

### Colony Formation Assay

After transfection of HepG2 and Huh7 cells, 5000 cells were plated into each well of 6-well plates and cultured for 14 days. Then, the cells were stained with 1% crystal violet staining solution (Beyotime, Shanghai, China) for 10 min according to the manufacturer's protocol. The colonies were counted with 50 cells under a microscope (Leica, Wetzlar, Germany).

### Autophagic Flux Detection Assay

The autophagic flux was detected using Autophagy indicator (HanBio, Shanghai, China) ac-

cording to the manufacturer's protocol. Briefly, HepG2 and Huh7 cells were transfected with mRFP-GFP-LC3B lentivirus for 48 h, the free red puncta and the merged yellow puncta were observed and counted under a confocal fluorescence microscope (UltraView Vox; PerkinElmer, Waltham, MA, USA).

### Luciferase Activity Assay

The sequences of lncRNA DANCR or ATG7 gene were amplified and cloned into pmirGLO vector, then, HepG2 and Huh7 cells were transfected with the recombinant plasmids and miRNA mimics using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h, Luciferase activity was determined by the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's protocol.

### Western Blot Assay

Total protein was extracted from cells using RIPA lysis buffer (Beyotime, Shanghai, China) containing with phenylmethanesulfonyl fluoride (PMSF). The total protein was quantified using BCA Protein Assay Kit (Beyotime, Shanghai, China) and separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, protein was transferred into polyvinylidene difluoride (PVDF) members (Millipore, Billerica, MA, USA) and blocked with 5% non-fat milk for 1 h. Next, the members were incubated with special primary antibodies against ATG7 (1:100000, ab52472), Beclin1 (1:1000, ab210498), p62 (1:10000, ab109012), LC3B (1:3000, ab51520), and GAPDH (1:10000, ab181602) overnight at 4°C. After that, the members were incubated with secondary goat anti-rabbit IgG H&L (HRP) antibody (1:4000, ab6721) for 1 h, and the bands were visualized using BeyoECL Plus (Beyotime, Shanghai, China) according to the manufacturer's protocol. All antibodies in this study were obtained from Abcam (Cambridge, MA, USA).

### Statistical Analysis

All statistics in this study were analyzed using SPSS 20.0 (SPSS Inc., Armonk, NY, USA) and GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA, USA). All data were presented as the means ± standard deviation (SD). The comparisons between two or multiple groups were carried out by Student's *t*-test or one-way ANOVA. Pairwise comparisons between groups were carried out by paired samples *t*-test. $p<0.05$ was considered significant different.

## Results

### Upregulation of LncRNA DANCR in HCC Tissues and Cell Lines

qRT-PCR was used to determine lncRNA DANCR expression in HCC tumor tissues and paired normal tissues. We found that lncRNA DANCR expression was significantly upregulated in HCC tissues compared to paired normal tissues (Figure 1A, B). Moreover, HCC patients with high expression of lncRNA DANCR showed lower survival rate than the patients with low expression of lncRNA DANCR (Figure 1C). Be-

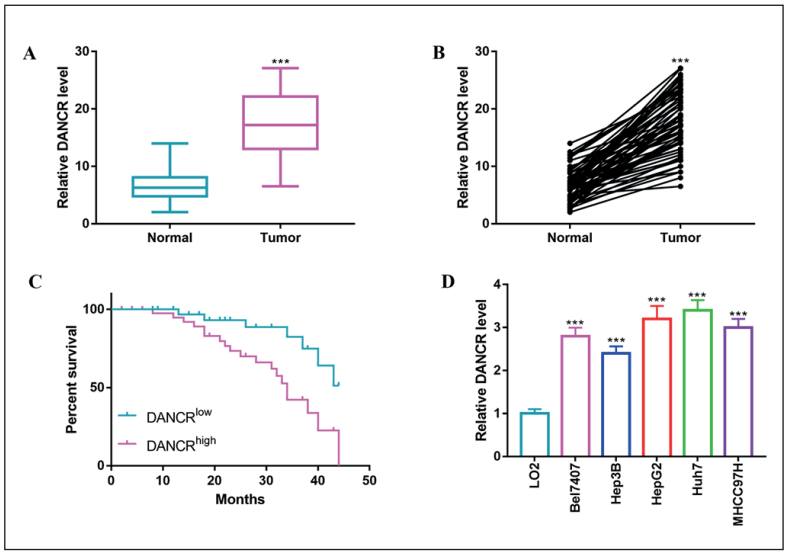


**Figure 1.** Upregulation of lncRNA DANCR in HCC tissues and cell lines. **A,** and **B,** The lncRNA DANCR expression in HCC tissues and paired normal tissues was detected by qRT-PCR (n=62). **C,** The survival curve of high (n=34) and low expression (n=28) of lncRNA DANCR in patients was analyzed using Kaplan-Meier methods. **D,** The lncRNA DANCR expression in HCC cell lines (Bel7407, Hep3B, HepG2, Huh7 and MHCC97H) and the hepatocyte cell line LO2 was measured by qRT-PCR. Data were represented as mean ± SD. ***$p<0.001$.

sides, the qRT-PCR results showed that lncRNA DANCR was strongly expressed in HCC cell lines (Bel7407, Hep3B, HepG2, Huh7 and MH-CC97H) compared to LO2 cell line (Figure 1D). These results indicate that lncRNA DANCR is highly expressed in HCC tissues and cell lines, and the HCC patients who have high expression of lncRNA DANCR exhibit poor survival rate.

### Knockdown of LncRNA DANCR Inhibits HCC Cell Proliferation in Vitro

We further detected the effects of lncRNA DANCR on HCC cell proliferation by silence of lncRNA DANCR *in vitro*. The knockdown efficiency of lncRNA DANCR was performed by qRT-PCR, and the si-DANCR-3 was chosen for subsequent experiments (Figure 2A, B). The cell viability results showed that HepG2 and Huh7 cell viability was suppressed by knockdown of lncRNA DANCR (Figure 2C, D). The colony formation results demonstrated that the knockdown of lncRNA DANCR dramatically inhibited the colony formation ability of HepG2 and Huh7 cells (Figure 2C, D). These data suggest that silence of lncRNA DANCR reduced HCC cell viability *in vitro*.

### Knockdown of LncRNA DANCR Suppresses Autophagy of HCC Cells in Vitro

Furthermore, we investigated the regulatory effects of lncRNA DANCR on autophagy in HCC. We found that red puncta, which presents autolysosomes, and the yellow puncta, which presents autophagosomes, were inhibited by knockdown of lncRNA DANCR, indicating the autophagic flux was inhibited by knockdown of lncRNA DANCR (Figure 3A, B). Besides, the Western blotting results showed that the protein levels of Beclin1, LC3-II, and p62 degradation were inhibited by the knockdown of lncRNA DANCR (Figure 3C). These results indicate that the silence of lncRNA DANCR inhibits autophagy of HCC cells *in vitro*.

### LncRNA DANCR Sponges MiR-222-3p, and MiR-222-3p Binds With ATG7

Next, we explored the mechanism of lncRNA DANCR in HCC. The downstream gene of lncRNA DANCR was predicted by StarBase, the binding site sequences of lncRNA DANCR on miR-222-3p were showed in Figure 4A. The Luciferase activity assay was used to evaluate

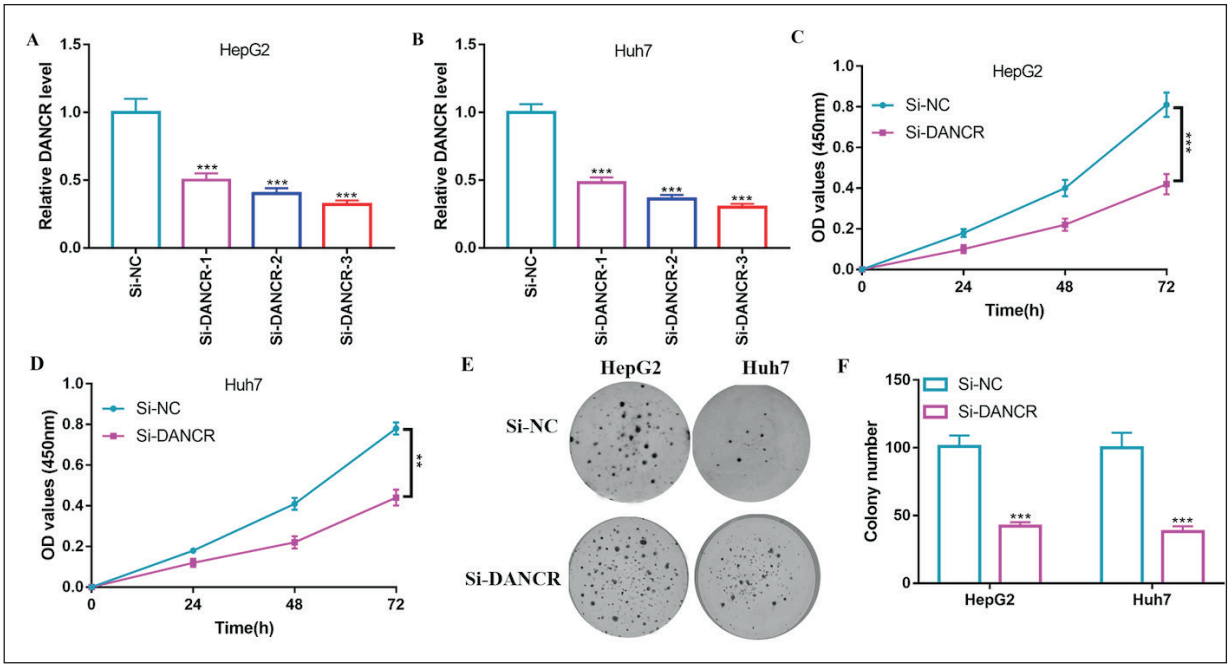


**Figure 2.** Knockdown of lncRNA DANCR inhibits HCC cell proliferation *in vitro*. **A,** and **B,** The lncRNA DANCR expression was evaluated by qRT-PCR after transfection with si-DANCR or si-NC in HepG2 and Huh7 cells. **C,** and **D,** Cell proliferation was determined by CCK-8 assay after transfection with si-DANCR or si-NC in HepG2 and Huh7 cells. **E,** and **F,** Cell colony formation was detected by colony formation assay after transfection with si-DANCR or si-NC in HepG2 and Huh7 cells. Data were represented as mean ± SD. ** $p<0.01$, *** $p<0.001$.

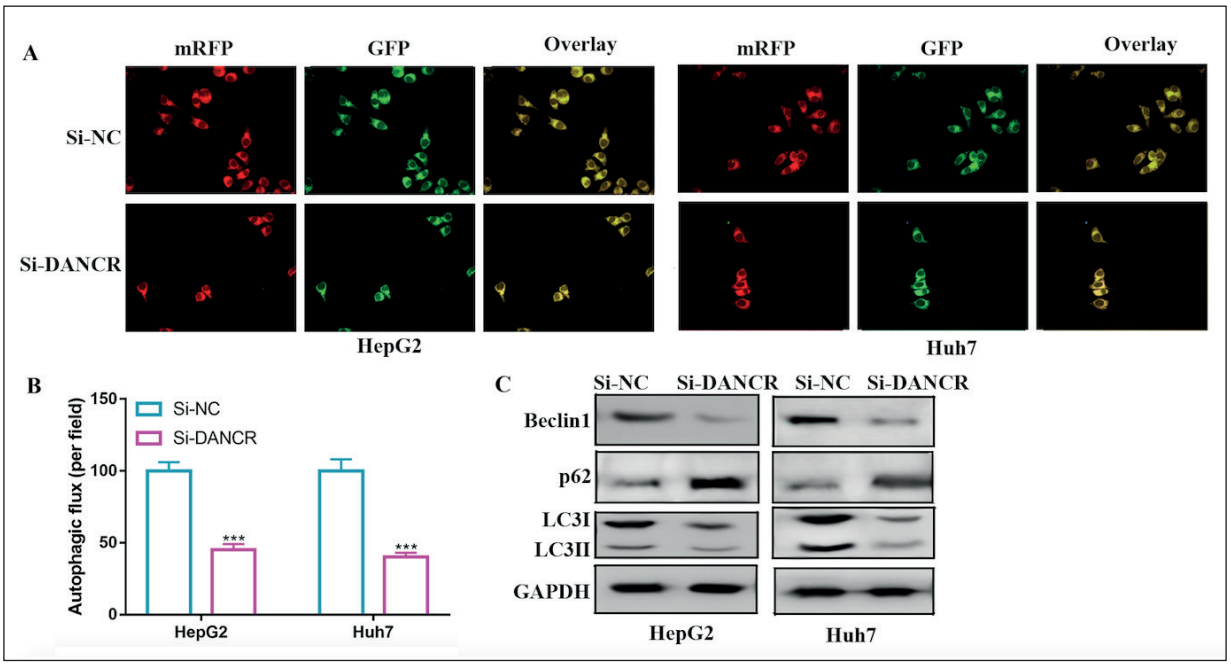


**Figure 3.** Knockdown of lncRNA DANCR suppresses autophagy of HCC cells *in Vitro*. **A,** and **B,** The autophagic flux was analyzed by mRFP-GFP-LC3B reporter after transfection with si-DANCR or si-NC in HepG2 and Huh7 cells (x 100). **C,** The Beclin1, p62, LC3II/I protein levels were detected by Western blotting after transfection with si-DANCR or si-NC in HepG2 and Huh7 cells. Data were represented as mean ± SD. *** $p<0.001$.

the relationship between lncRNA DANCR and miR-222-3p; the results showed that the Luciferase activity of DANCR-wt was reduced, but the Luciferase activity of DANCR-mut was not significantly influenced by miR-222-3p mimics (Figure 4B, C). Besides, the expression of lncRNA DANCR in HepG2 and Huh7 cells was reduced by miR-222-3p mimics (Figure 4D). The miR-222-3p expression was strongly downregulated in HCC tissues compared to normal tissues (Figure 4E), and dramatically reduced in HCC cell lines (Bel7407, Hep3B, HepG2, Huh7 and MHCC97H) compared to LO2 cells (Figure 4F). Next, the target gene of miR-222-3p was prediated by StarBase and Targetscan (Figure 4G), and the prediction demonstrated that the Luciferase activity of ATG7-wt was significantly inhibited, but the Luciferase activity of ATG7-mut was not changed by miR-222-3p mimics (Figure 4H, I). Both the mRNA and protein levels of ATG7 were inhibited by miR-222-3p mimics (Figure 4J, K). We also found that the mRNA levels of AGT7 were enhanced in HCC tissues and cell lines (Figure 4L, M). These results suggest that lncRNA DANCR directly interacts with miR-222-3p, the ATG7 is the downstream target gene of miR-222-3p in HCC cells.

### *LncRNA DANCR Accelerates HCC Cell Proliferation through Promoting ATG7 Via Sponging MiR-222-3p*

We further investigated the mechanism of lncRNA DANCR-induced cell proliferation through gain of lncRNA DANCR function. We found that the HepG2 and Huh7 cell proliferation was enhanced by lncRNA DANCR upregulation, but lncRNA DANCR function was repressed by miR-222-3p mimics and rescued by ATG7 upregulation (Figure 5A, B). The colony formation results also showed that the colony formation ability was elevated by lncRNA DANCR upregulation, but lncRNA DANCR function was inhibited by miR-222-3p mimics and rescued by ATG7 upregulation (Figure 5C, D). Our data indicate that lncRNA DANCR promotes HCC cell proliferation and colony formation by upregulating ATG7 expression *via* suppressing miR-222-3p.

### *LncRNA DANCR Accelerates Autophagy of HCC Cells through Promoting ATG7 Via Sponging MiR-222-3p*

Next, we confirmed the mechanism of lncRNA DANCR-induced autophagy through gain of lncRNA DANCR function. The results showed that the autophagic flux was induced by lncRNA

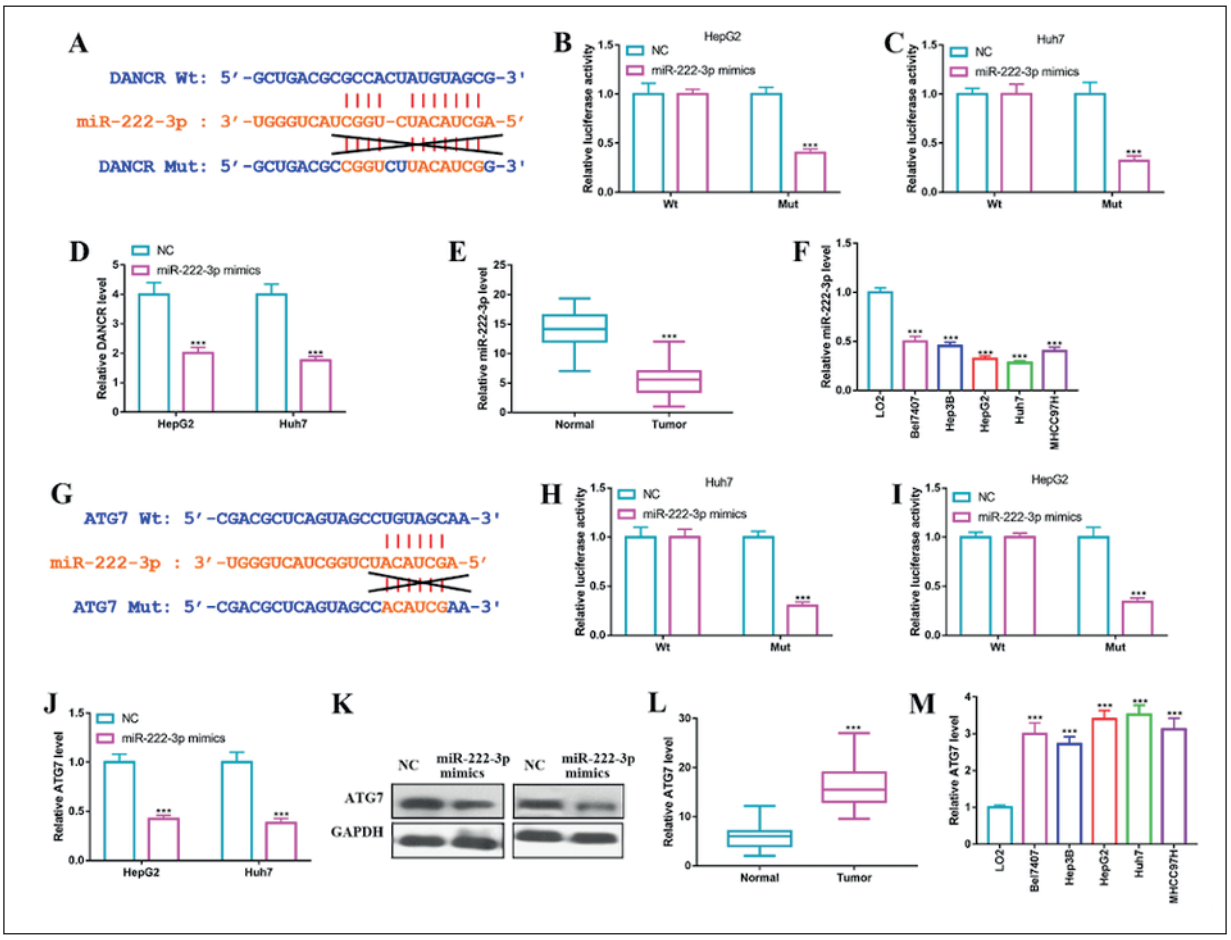


**Figure 4.** LncRNA DANCR sponges miR-222-3p, and miR-222-3p binds with ATG7. **A,** The binding sites of lncRNA DANCR on miR-222-3p were predicated by StarBase. **B,** and **C,** The Luciferase activity was evaluated by dual-luciferase reporter system after co-transfection with DANCR-wt or DANCR-mut reporter and miR-222-3p mimics in HepG2 and Huh7 cells. **D,** The lncRNA DANCR expression was evaluated by qRT-PCR after transfection with miR-222-3p mimics in HepG2 and Huh7 cells. **E,** The miR-222-3p expression in HCC tissues and paired normal tissues was detected by qRT-PCR (n=62). **F,** The miR-222-3p expression in HCC cell lines (Bel7407, Hep3B, HepG2, Huh7 and MHCC97H) and the hepatocyte cell line LO2 was measured by qRT-PCR. **G,** The binding sites of ATG7 on miR-222-3p were predicated by StarBase and Targetscan. **H,** and **I,** The Luciferase activity was evaluated by Dual-Luciferase reporter system after co-transfection with ATG7-wt or ATG7-mut reporter and miR-222-3p mimics in HepG2 and Huh7 cells. **J,** and **K,** The ATG7 mRNA and protein expression was analyzed by qRT-PCR and Western blotting after transfection with miR-222-3p mimics in HepG2 and Huh7 cells. **L,** The ATG7 expression in HCC tissues and paired normal tissues was detected by qRT-PCR (n=62). **M,** The ATG7 expression in HCC cell lines (Bel7407, Hep3B, HepG2, Huh7 and MHCC97H) and the hepatocyte cell line LO2 were measured by qRT-PCR. Data were represented as mean ± SD. *** $p<0.001$.

DANCR upregulation, but lncRNA DANCR function was blocked by miR-222-3p mimics and rescued by ATG7 upregulation (Figure 6A, B). Besides, the protein levels of ATG7, Beclin1, LC3-II, and the p62 degradation were enhanced by lncRNA DANCR upregulation, but lncRNA DANCR function was alleviated by miR-222-3p mimics and rescued by ATG7 upregulation (Figure 6C, D). These results indicate that lncRNA DANCR induces autophagy by increasing ATG7 by inhibiting miR-222-3p.

## Discussion

LncRNAs have important roles in HCC by regulating a wide range of biological processes. For example, lncRNA DUXAP8 contributes to HCC progression by accelerating proliferation, metastasis and epithelial-mesenchymal transition of HCC cells[18]. LINC00152 induces proliferation, migration and invasion of HCC cells to promote HCC progression[26]. LncRNA GMAN promotes HCC progression by suppressing

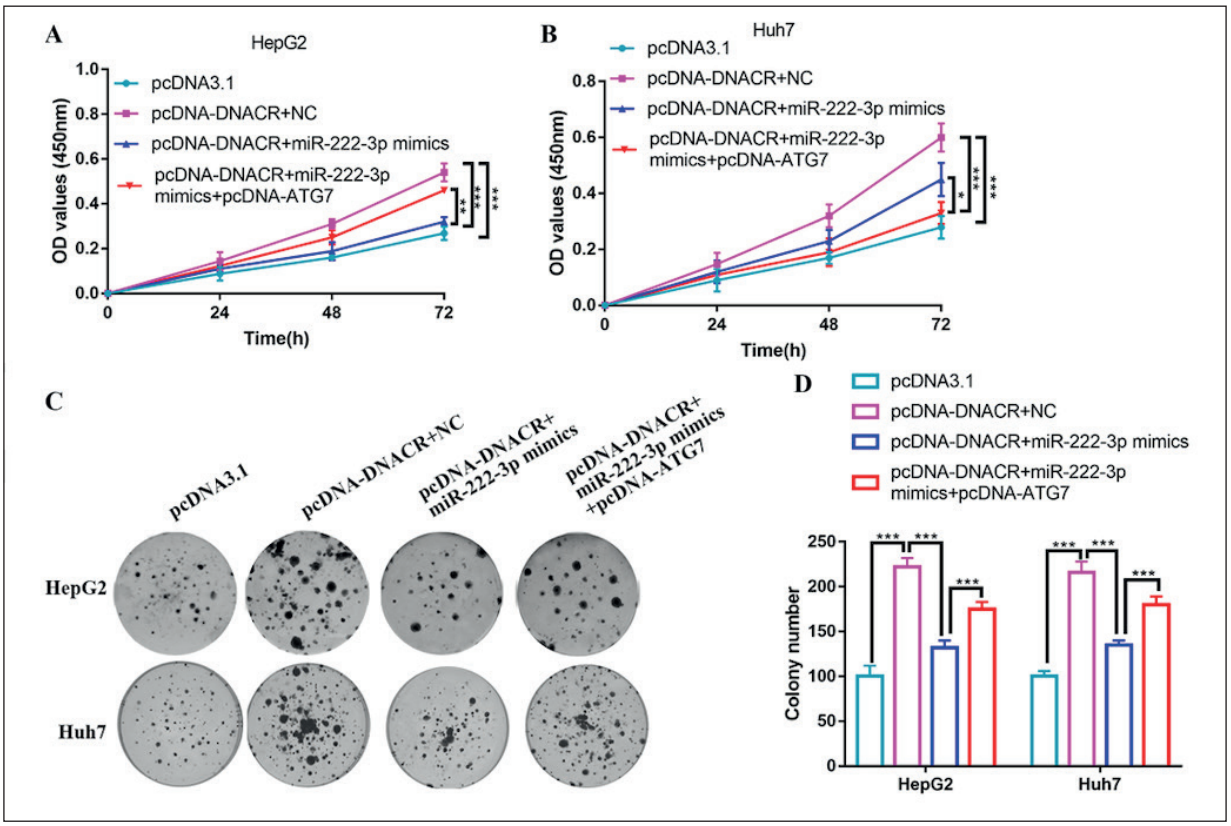


**Figure 5.** LncRNA DANCR Accelerates HCC cell proliferation through promoting ATG7 via sponging miR-222-3p. **A,** and **B,** Cell proliferation was determined by CCK-8 assay after transfection with pcDNA-DNACR, or pcDNA-ATG7, or pcDNA-3.1, or miR-222-3p mimics in HepG2 and Huh7 cells. **C,** and **D,** Cell colony formation was detected by colony formation assay after transfection with pcDNA-DNACR, or pcDNA-ATG7, or pcDNA-3.1, or miR-222-3p mimics in HepG2 and Huh7 cells. Data were represented as mean ± SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

apoptosis and inducing survival of HCC cells[27]. Besides, LINC00665 contributes to the progression and development of HCC by induction of cell proliferation and autophagy and inhibition of apoptosis[28].

Here, we found that the elevation of lncRNA DANCR in HCC tissues and cell lines, and knockdown of lncRNA DANCR inhibited cell proliferation and autophagy. Previous studies reported that lncRNA DANCR acts as an important oncogene in most malignant cancers, including ovarian cancer[29], esophageal squamous cell carcinoma[30], cholangiocarcinoma[29], HCC[24], and osteosarcoma[25] by regulating cell proliferation, migration, EMT, angiogenesis, autophagy and so on. In this study, we firstly proved that lncRNA DANCR facilitated the progression of HCC by promoting cell proliferation and autophagy.

Autophagy is an evolutionarily conservative process of catabolism that regulates cell survival and death by modulating autophagy-associated genes to response stress, including hypoxia, nutrient deficiency and energy exhaust[31]. Previous studies have demonstrated that autophagy has a tumor promoting role in HCC, such as, lncRNA HCG11 promotes the progression of HCC by acceleration of autophagy[10], and induction of autophagy enhances the progression of HCC and HCC cells resistance to sorafenib[32]. Here, we found that lncRNA DANCR promoted proliferation and autophagy of HCC cells by upregulating ATG7 and sponging miR-222-3p.

ATGs act as the vital roles in autophagosome formation and autophagic cargo delivery to the lysosome[33]. In most cases, lncRNAs regulate tumorigenesis and progression by regulating the transcriptional and post-transcriptional of ATGs. For example, lncRNA MALAT1 induces autophagy in gastric cancer by upregulating ATG5 expression[34]. In addition, lncRNA KCNQ1OT1 facilitates the progression of non-small cell lung cancer *via* activating autophagy and increasing ATG3[35].

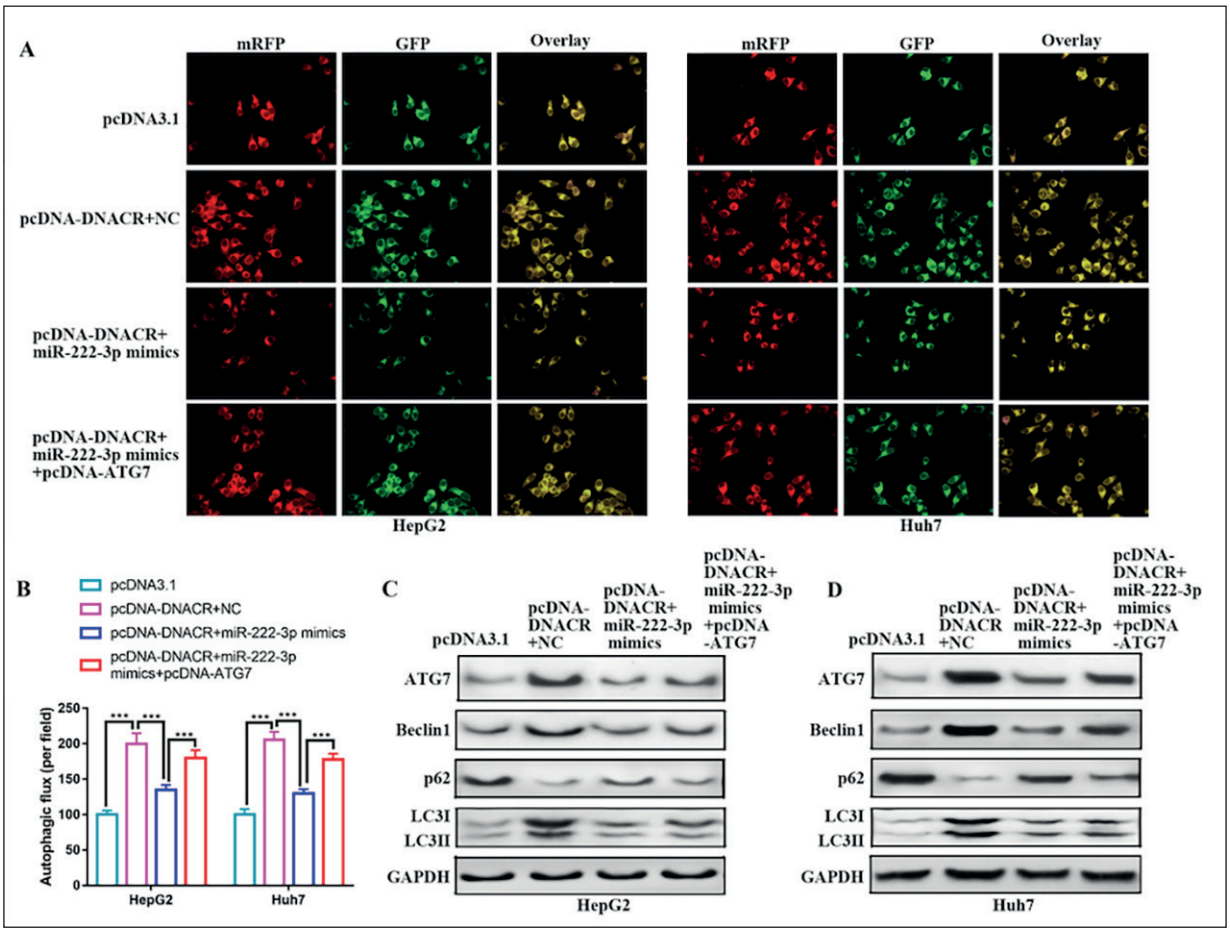


**Figure 6.** LncRNA DANCR accelerates autophagy of HCC cells through promoting atg7 via sponging miR-222-3p. **A,** and **B,** The autophagic flux was analyzed by mRFP-GFP-LC3B reporter after transfection with pcDNA-DNACR, or pcDNA-ATG7, or pcDNA-3.1, or miR-222-3p mimics in HepG2 and Huh7 cells (100×). **C,** The ATG7, Beclin1, p62, LC3II/I protein levels were detected by Western blotting after transfection with pcDNA-DNACR, or pcDNA-ATG7, or pcDNA-3.1, or miR-222-3p mimics in HepG2 and Huh7 cells. Data were represented as mean ± SD. *** $p<0.001$.

LncRNA HCG11 induces autophagy in HCC by accelerating ATG12 expression[10]. It also reported that lncRNA CCAT1 activates autophagy in HCC by upregulating ATG7[36]. ATG7 is an E1 enzyme that activates ATG8 and ATG12, and deliveries ATG8 and ATG12 to cognate E2 enzyme ATG3 or ATG10, and ATG7 is a part of ATG7-ATG8-ATG3 complex, which regulates autophagosome biogenesis and recruits the cargos of autophagy[37]. In our study, we found that lncRNA DANCR upregulated the mRNA and protein expression of ATG7 by interacting with miR-222-3p.

## Conclusions

Our study demonstrates the regulatory role of lncRNA DANCR in promoting the progression of HCC and elucidates the mechanism of lncRNA DANCR in regulating proliferation and autophagy of HCC cells. Our data show that lncRNA DANCR facilitates proliferation and autophagy by activating ATG7 expression *via* sponging miR-222-3p in HCC. Our research provides a novel viewpoint and therapeutic strategy for HCC treatment by repressing the HCC cell proliferation and autophagy by targeting lncRNA DANCR.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

### Consent for Publication

All authors in this research agreed to publish the manuscript.

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