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Original Article

Downregulated miR-621 promotes cell proliferation via targeting CAPRIN1 in hepatocellular carcinoma

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Abstract: MicroRNAs (miRNAs) have been reported to play an essential role in tumor development and progression. However, the function of miR-621 in hepatocellular carcinoma (HCC) remains largely unexplored. In this study, we found that miR-621 was downregulated in the HCC specimens and cell lines, and lower expression of miR-621 indicated poor survival. Overexpression of miR-621 was shown to induce G0/G1 cell cycle arrest and inhibit cell proliferation *in vitro* and *in vivo*. Luciferase assays revealed that cell cycle-associated protein 1 (CAPRIN1) is a novel functional downstream target of miR-621. miR-621 could regulate c-MYC and cyclin D2 expression by directly targeting CAPRIN1. Further study revealed that CAPRIN1 was upregulated in the HCC specimens and cell lines. Restoration of CAPRIN1 neutralized the miR-621-induced cell cycle arrest and cell proliferation inhibition. Taken together, our findings suggest that miR-621 acts as a tumor suppressor gene in HCC progression by downregulating CAPRIN1 expression and could be a novel potential diagnostic and prognostic biomarker for HCC.

Keywords: HCC, miR-621, CAPRIN1, proliferation

Introduction

Hepatocellular carcinoma (HCC) is one of the most deadly malignancies and the second leading cause of tumor-related death worldwide [1, 2]. During the past decade, despite advanced therapeutic options, including tumor resection, liver transplantation, radiotherapy and chemotherapy, the prognosis of patients with HCC remains poor due to the high frequency of recurrence and metastasis [3-5]. Hence, elucidating the pathogenic mechanisms involved in HCC and finding some potential therapeutic targets for HCC are urgently needed.

MicroRNAs (miRNAs) are a class of noncoding small RNA molecules that can bind to the 3'-untranslated regions (3' UTRs) of target genes, resulting in translational repression and gene silencing [6, 7]. Previous investigations have demonstrated the vital function of miRNAs in diverse biological processes, including proliferation, cell cycle, apoptosis, differentiation, metastasis and metabolism [8-10]. Dysregulation of miRNAs in HCC has been revealed to promote tumorigenicity by upregulating oncogene expression or downregulating tumor suppressor genes [11-13]. Previous genome-wide studies have indicated that deletions at the chromosome (chr) 13q region are frequent in HCC, implicating this region in liver carcinogenesis [14, 15]. However, the genes within this region are largely unknown. A microRNA gene, miR-621, is located in this region. Recently, it was found that miR-621 sensitizes breast cancer to chemotherapy by targeting FBXO11 [16]. Later, studies have demonstrated that upregulated miR-621 inhibits proliferation and metastasis and promotes apoptosis in breast cancer cell lines [17]. However, the function of miR-621 in HCC is still unclear.

Our research has demonstrated the downregulation of miR-621 in HCC tissues and cell lines.

Table 1. Primer sequences used in qRT-PCR

Symbol	Primer	Sequence (5'→3')
CAPRIN1	Forward Primer	TCTCGGGGTGATCGACAAGAA
	Reverse Primer	CCCTTTGTTCATTCGTTCCTGG
FBXO11	Forward Primer	GATGGACGAGGCCTTATTGA
	Reverse Primer	TGTTATGCCGAACAATTGGA
c-MYC	Reverse Primer	GGCTCCTGGCAAAAGGTCA
	Reverse Primer	CTGCGTAGTTGTGCTGATGT
CCND2	Forward Primer	ACCTTCCGCAGTGCTCCTA
	Reverse Primer	CCCAGCCAAGAAACGGTCC
β-actin	Forward Primer	AGCGAGCATCCCCCAAAGTT
	Reverse Primer	GGGCACGAAGGCTCATCATT

HCC patients with higher miR-621 expression showed a significantly longer overall survival time. Furthermore, miR-621 overexpression can inhibit the proliferation rate and induce cell cycle arrest at the G1 phase by targeting CAPRIN1 and affecting its expression. Thus, our results revealed miR-621 as a tumor suppressor miRNA in HCC via downregulation of CAPRIN1, and miR-621 may be a novel diagnostic and prognostic biomarker for HCC.

Materials and methods

Tissue specimens and cell lines

We obtained HCC cells (SMMC-7721, MHCC-97L, MHCC-LM3, and Huh7) and immortalized human hepatocyte LO2 cells from American Type Culture Collection (ATCC, USA). Cells used in this study were grown in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin (Invitrogen, Carlsbad, CA) in a humidified incubator (stabilized at 5% CO_2 and 37°C). We obtained 56 paired tumor and adjacent normal tissues from patients who were diagnosed with HCC and underwent surgical resection at the First Affiliated Hospital of Nanjing Medical University, China. Before the collection of specimens, we obtained consent from the patients or their relatives. The current study was approved by the Institutional Ethics Board of the First Affiliated Hospital of Nanjing Medical University.

RNA isolation and real-time qPCR analysis

Following the manufacturer's instructions, total RNA was extracted from HCC tissues and cells using TRIzol reagent (Invitrogen, USA). Complementary DNA (cDNA) was reverse transcribed using a PrimeScript RT Reagent kit with gDNA Eraser (TaKaRa, RR047A) according to the manufacturer's instructions. We obtained bulge-loopTM miRNA qRT-PCR primer sets specific for miR-621 and U6 from RiboBio (Guangzhou, China). The expression level of the specific transcripts was normalized to internal controls (β-actin or U6), and the results were calculated using the $2^{-\Delta\Delta CT}$ method. The specific primers of the target mRNA and internal control are shown in **Table 1**.

Cell treatment

LV-hsa-miR-621-mimic (pre-miR-621), LV-hsa-miR-621-inhibitor (miR-621-inhibitor), LV-miR-621-NC, LV-CAPRIN1, and LV-CAPRIN1-NC constructed in lentiviral vectors were purchased from GenePharma (Shanghai, China). SMMC-7721 and MHCC-97L cells were transfected with lentiviral vectors at an appropriate multiplicity of infection (MOI). Stably transfected cells were selected by puromycin according to protocols.

CCK-8 cell proliferation assay

Following the manufacturer's instructions, we evaluated the effect of miR-621 on cell growth with a Cell Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan). Transfected cells were incubated in 96-well plates at a density of 1×10^3 cells per well and cultured as previously described. Ten microliters of CCK-8 solution was mixed with serum-free medium and added to each well every 24 h. After 2 h of incubation, we detected the absorbance at 450 nm in a microplate reader.

Colony formation assay

To evaluate the colony formation ability, we seeded 500 cells per well into 6-well plates and cultured as described before. After 10 days, the cells were fixed with ethyl alcohol for 30 s and stained with 1% crystal violet for 15 min. Individual colonies representing cell growth were counted by eye after washing with phosphate buffer saline (PBS).

5-ethynyl-2'-deoxyuridine (Edu) proliferation assay

An EdU incorporation assay kit (Ribobio, China) was used to evaluate the cell proliferation abil-

ity. Briefly, cells (2×10^4) were incubated with EdU for 2 h. Then, cells were neutralized with 2 mg/ml glycine and permeabilized in 0.5% Triton X-100 for 20 min. After extensive washing with PBS, cells were incubated with Apollo staining reaction buffer for 30 min. Subsequently, the nuclei of cells were stained with DAPI for 15 min, and the EdU incorporation rate was analyzed with a fluorescence microscope.

Flow cytometric analysis

For cell cycle analysis, transfected cells seeded in 6-well plates were digested and fixed overnight with 70% ethanol at 4°C. After being resuspended in 500 μL of PBS, cells were stained with 500 μL PI staining solution (BD Pharmingen, USA) for 30 min. Then, the stained cells were analyzed by a BD FACSCanto II (BD Biosciences, USA) flow cytometer. The percentage of the cells in G1, S, and G2/M phase was analyzed using ModFit software.

Western blot analysis

Proteins of HCC tissues and cell lines were separated based on their molecular weight on 10% SDS-PAGE gels and then transferred to PVDF membranes. The membranes were incubated with the appropriate primary antibody overnight at 4°C. Antibodies against CAPRIN1 (HPA018-126, Atlas Antibodies), FBXO11 (ab181801, Abcam), CCND2 (ab181150, Abcam), and c-MYC (ab32072, Abcam) were used, and GAPDH (ab8245, Abcam) was used as an internal control. Protein expression levels were detected by ECL Plus (EMD Millipore, Billarica, MA, USA).

Fluorescence in situ hybridization (FISH)

We performed FISH to evaluate miR-621 expression in HCC tissues and matched normal tissues. The miR-621 antisense oligonucleotide probes were purchased from QingKe (Beijing, China). The FISH assay was conducted as previously described [18].

Immunohistochemical staining

HCC tissues were fixed in 4% paraformaldehyde, embedded with paraffin and cut into 5-μm-thick sections. The sections were incubated with the primary antibody (CAPRIN1, HPA018126, Atlas Antibodies; Ki-67, Maixin Bio, China). After incubation with secondary antibody at 37°C for 1 h, the sections were incubated with 3,3'-diaminobenzidine solution for 3 min, and hematoxylin was used as a light nuclear counterstain. The tumor sections were evaluated in a blinded manner.

Dual-luciferase reporter assay

The potential binding sites of miR-621 in the CAPRIN1 3' UTR were predicted by TargetScan7.2, miRDB, and TargetMiner. Wild-type (WT) or mutant (MUT) CAPRIN1-3' UTR sequences designed by Genescript (Nanjing, China) were inserted within the pGL3 plasmid (Ambion, Austin, TX, USA). We seeded 5×10^5 cells per well into a 24-well plate the day before transfection. Then, luciferase reporter plasmids were cotransfected with the miR-621 mimic or negative control using Lipofectamine 3000 (Invitrogen). Luciferase activities were measured at 24 h after transfection using the Dual-Luciferase Reporter Assay System (Promega, USA).

Tumor xenograft in animals

All procedures involving animals were approved by the Experimental Animal Ethical Committee of Nanjing Medical University. Twenty BALB/c mice (aged 4-6 weeks) were purchased from the Model Animal Research Center of Nanjing Medical University (Nanjing, China) and randomly divided into 4 groups. Nude mice were injected with 1×10^6 MHCC-97L cells transfected with miR-621-NC/miR-621-inhibitor × and SMMC-7221 cells transfected with miR-621-NC/pre-miR-621 into the left inguinal region. We recorded the tumor size every 3 days and euthanized the mice 30 days later. The formula used to calculate the tumor volume was as follows: volume = $1/2 \times \text{length} \times \text{width}^2$.

Statistical analysis

Data are shown as the mean ± standard error of the mean (S.E.M.). Each experiment was repeated at least three times. Statistical analysis was performed using GraphPad Prism software (version 5.0; San Diego, CA, USA). The chi-squared test was adopted to evaluate the relationship between miR-621 expression level and clinical parameters. A spearman correlation test was used for correlation analysis between miR-621 and CAPRIN1. The data were considered statistically significant at $P < 0.05$ as indicated by '*'; $P < 0.01$ as indicated by '**'; and $P < 0.001$ as indicated by '**'.

Results

miR-621 expression is downregulated in HCC tissues and cell lines

To investigate the dysregulation of miR-621 in HCC tissues, we obtained 56 pairs of HCC tissues and adjacent normal tissues. By qRT-PCR, we found that the expression level of miR-621 in tumors was significantly lower than that in adjacent normal tissues (**Figure 1A**). Moreover, we found that miR-621 expression was lower in HCC cells (SMMC-7721, MHCC-97L, MHCC-LM3, Huh7) compared to immortalized human hepatocyte LO2 cells (**Figure 1B**). Then, we randomly chose four pairs of HCC and adjacent noncancerous tissues for FISH analysis and found lower miR-621 expression in the HCC tissues (**Figure 1C**). To evaluate the clinicopathological significance of miR-621, patients involved in this study were divided into high and low expression groups based on the median level of miR-621. As indicated in **Table 2**, lower miR-621 expression was significantly associated with larger tumor size ($P < 0.05$). In addition, Kaplan-Meier analysis revealed that low miR-621 expression correlated with poor overall survival (**Figure 1D**, $P < 0.01$).

miR-621 inhibits HCC cell growth and induces cell cycle arrest

The HCC cell line SMMC-7721, which exhibited the lowest expression level of miR-621, was transfected with pre-miR-621, while MHCC-97L cells, which exhibited the highest expression level of miR-621, were transfected with the miR-621 inhibitor. qRT-PCR confirmed the transfection efficiency. As shown in **Figures 1E** and S1, cells treated with pre-miR-621 presented a marked increase in the miR-621 expression level, while transfection with miR-621 inhibitor notably inhibited the miR-621 expression level compared to that in the negative control group. CCK-8 assay, EdU assay and colony formation assay were conducted to evaluate the function of miR-621 in cell proliferation. As indicated in **Figure 2A**, CCK-8 assays revealed that overexpression of miR-621 suppressed the growth of SMMC-7721 cells, while knockdown of miR-621 had the opposite effects on MHCC-97L cells. Meanwhile, ectopic expression of miR-621 suppressed the colony formation ability of SMMC-7721 cells. In contrast, knockdown of miR-621 promoted colony formation in MHCC-97L cells (**Figure 2B**). The number of SMMC-7721 cells incorporating EdU in the pre-miR-621 group decreased significantly compared with that in the miR-NC group, while the MHCC-97L cells transfected with the miR-621-inhibitor showed the opposite trend (**Figure 2C**).

As miR-621 remarkably inhibited HCC cell proliferation, we next investigated the function of miR-621 in the regulation of the cell cycle. We performed flow cytometry to examine the effects of miR-621 dysregulation on the cell cycle, and the results are shown in **Figure 2D**. We observed that overexpression of miR-621 in the SMMC-7721 cells presented a remarkable increase in G0/G1 phase, while knockdown of miR-621 in the MHCC-97L cells led to a marked increase in S phase. Thus, these data demonstrated that overexpressed miR-621 inhibited HCC cell growth and induced G0/G1 cell cycle arrest.

CAPRIN1 is upregulated in human HCC tissues and cell lines

To further explore the mechanism of miR-621-induced cell proliferation inhibition, bioinformatics databases (TargetScan, miRDB and TargetMiner) were utilized to predict the potential downstream target of miR-621 (**Figure 3A**). A putative binding site for miR-621 was identified in the 3' UTR of CAPRIN1. A previous study revealed that CAPRIN1 was highly expressed in HCC tissues and closely associated with poor prognosis [19]. qRT-PCR and western blotting were performed to evaluate CAPRIN1 expression in HCC cells and LO2 cells. As shown in **Figure 3B**, CAPRIN1 was upregulated in the HCC cell lines. Furthermore, qRT-PCR was conducted to examine the relative expression of CAPRIN1 in 56 pairs of HCC tissues and matched noncancerous tissues. Interestingly, we found increased expression of CAPRIN1 in the HCC tissues (**Figure 3C**). Immunohistochemistry further confirmed this result (**Figure 3E**). In addition, Kaplan-Meier analysis indicated that higher CAPRIN1 expression was correlated with a remarkably shorter overall survival time (**Figure 3D**). Further experiments revealed that CAPRIN1 knocking down inhibited HCC cell proliferation and induced cell cycle arrest. The results were shown in Figures S2 and S3.

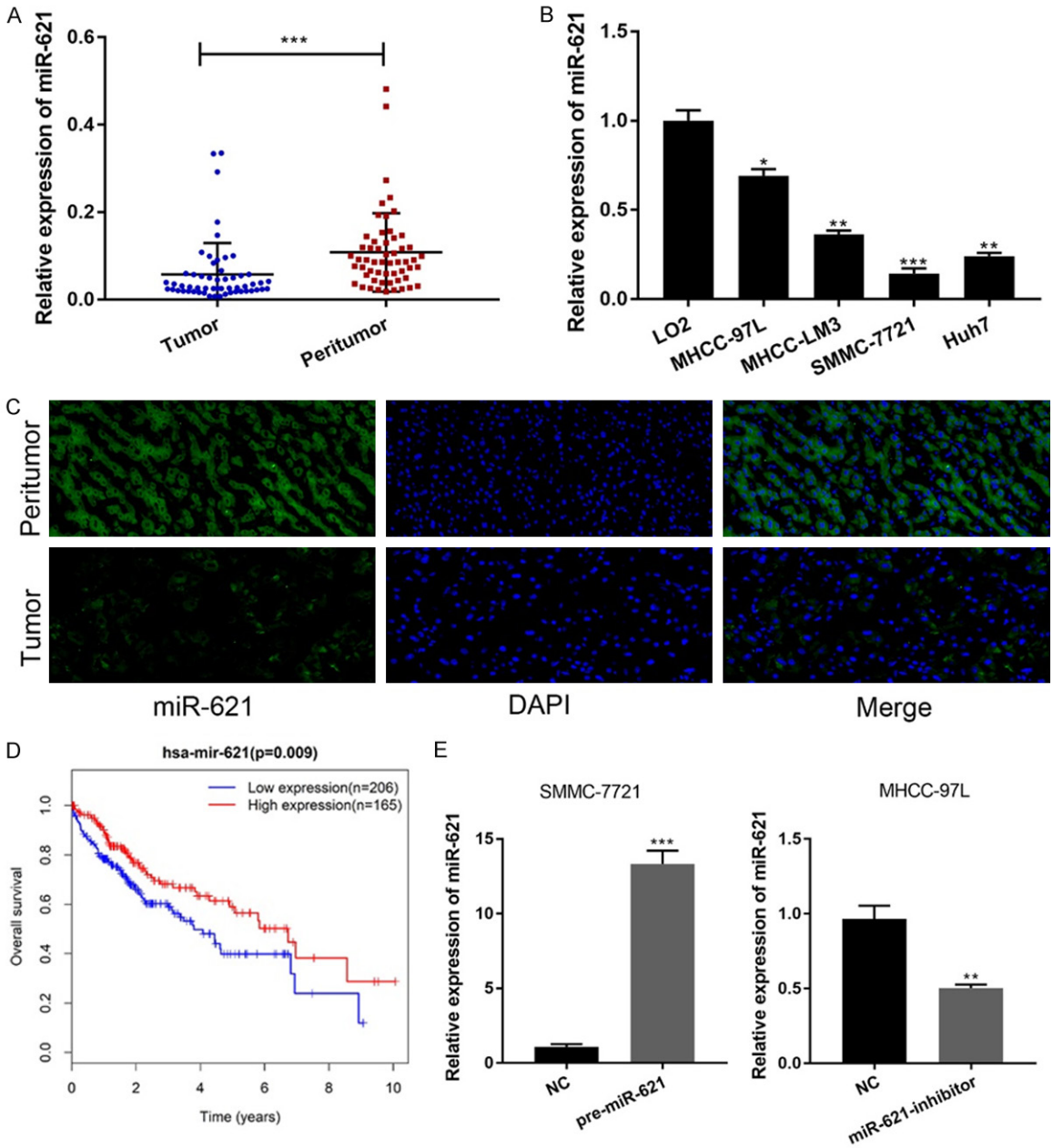

Figure 1. The miR-621 expression level is decreased in the HCC tissue samples and cell lines. A. qRT-PCR was used to examine the expression of miR-621 in 56 pairs of HCC tissues and corresponding peritumor tissues. B. The expression levels of miR-621 in the HCC cell lines and normal LO2 cells. C. The expression of miR-621 in the HCC specimens and adjacent normal tissues was detected by FISH assay (scale bars, 50 μm). D. Kaplan-Meier analysis for overall survival of patients with HCC according to the miR-621 expression in all patients (TCGA datasets). E. The SMMC-7721 and MHCC-97L cells were transfected with lentivirus overexpressing miR-621 (defined as pre-miR-621) or lentivirus with short hairpin RNA targeting miR-621 (defined as miR-621-inhibitor). The negative control (NC) cells included a nontargeting sequence. miR-621 expression levels were analyzed by qRT-PCR. All data are presented as the mean ± S.E.M. (*P < 0.05, **P < 0.01, and ***P < 0.001).

miR-621 directly targets CAPRIN1 in HCC cells

In the preceding study, we determined the miR-621 and CAPRIN1 expression in 5 different cell lines via qRT-PCR (**Figures 1B** and **3B**). The results indicated that miR-621 was expressed at a low level, while CAPRIN1 expression levels were increased compared to those observed in LO2 cells. In addition, CAPRIN1 expression was negatively associated with miR-621 in 56 pairs

Table 2. Association between miR-621 expression and clinicopathologic features of patients with hepatocellular carcinoma (n = 56)

Characteristics	Number	Low miR-621	High miR-621	*P* value
Age (years)				0.590
≤ 60	24	13	11	
> 60	32	15	17	
Gender				0.577
Female	20	9	11	
Male	36	19	17	
Liver cirrhosis				0.445
No	8	5	3	
Yes	48	23	25	
HBsAg status				0.763
Negative	15	7	8	
Positive	41	21	20	
α-fetoprotein (ng/ml)				0.280
≤ 20	24	10	14	
> 20	32	18	14	
Tumor size (cm)				0.016*
≤ 5	29	10	19	
> 5	27	18	9	
Tumor multiplicity				0.585
Single	34	16	18	
Multiple	22	12	10	
Edmondson grade				0.783
I-II	35	17	18	
III-IV	21	11	10	
Tumor-node-metastasis stage				0.280
I-II	32	18	14	
III-IV	24	10	14	

*Indicates P < 0.05.

of HCC tissues (**Figure 4A**). Further experiments demonstrated that upregulation of miR-621 resulted in decreased CAPRIN1 transcription and protein levels, while downregulated miR-621 led to the opposite results (**Figure 4B** and **4C**). Finally, a dual-luciferase reporter assay was conducted to verify whether CAPRIN1 was a direct target of miR-621. Wild-type (WT) or mutant (MUT) CAPRIN1-3' UTR were inserted into a luciferase reporter vector. Our results demonstrated that miR-621 led to decreased luciferase activity of the WT-CAPRIN1-3' UTR, whereas the luciferase activity of the MUT-CAPRIN1-3' UTR was almost unchanged (**Figure 4D** and **4E**). Overall, miR-621 can directly and negatively regulate CAPRIN1.

Restoration of CAPRIN1 reverses the effects of miR-621 on HCC cells

To investigate whether CAPRIN1 was involved in the antitumor effects of miR-621 in HCC, we transfected SMMC-7721 cells with pre-miR-621 lentivirus. After 72 h, the cells were transfected with LV-CAPRIN1. Upregulation of CAPRIN1 was corroborated by qRT-PCR and western blot (**Figure 5E** and **5F**). CCK-8, EdU and colony formation assays confirmed that CAPRIN1 restoration reversed the proliferation-inhibiting effect of miR-621 (**Figure 5A-C**). In addition, flow cytometric analysis revealed that overexpression of CAPRIN1 could abolish cell cycle arrest caused by upregulated miR-621 in the SMMC-7721 cells (**Figure 5D**). Taken together, the data show that restoration of CAPRIN1 could neutralize the influence of miR-621 on HCC cell lines.

miR-621 regulates CCND2/c-MYC by targeting CAPRIN1

Previous studies have shown that CAPRIN1 regulates cell proliferation and the cell cycle by binding to c-MYC and cyclin D2 mRNA [20, 21]. In this study, we confirmed that miR-621 can directly target CAPRIN1. Thus, we wondered whether miR-621 could regulate the expression levels of CCND2 and c-MYC. After transfection with pre-miR-621 and miR-621-inhibitor lentivirus, the expression levels of CCND2 and c-MYC were evaluated by qRT-PCR and western blot. Cells overexpressing miR-621 showed a clear attenuation of CCND2 and c-MYC protein expression, whereas inhibition of miR-621 in the MHCC-97L cells showed a moderate increase in CCND2 and c-MYC protein expression (**Figure 5E**). The mRNA expression levels of CCND2 and c-MYC tended to be similar (**Figure 5F**). Furthermore, rescue experiments were performed. As indicated in **Figure 5F**, suppressed expression levels of CCND2 and c-MYC were restored. In summary, we determined that miR-

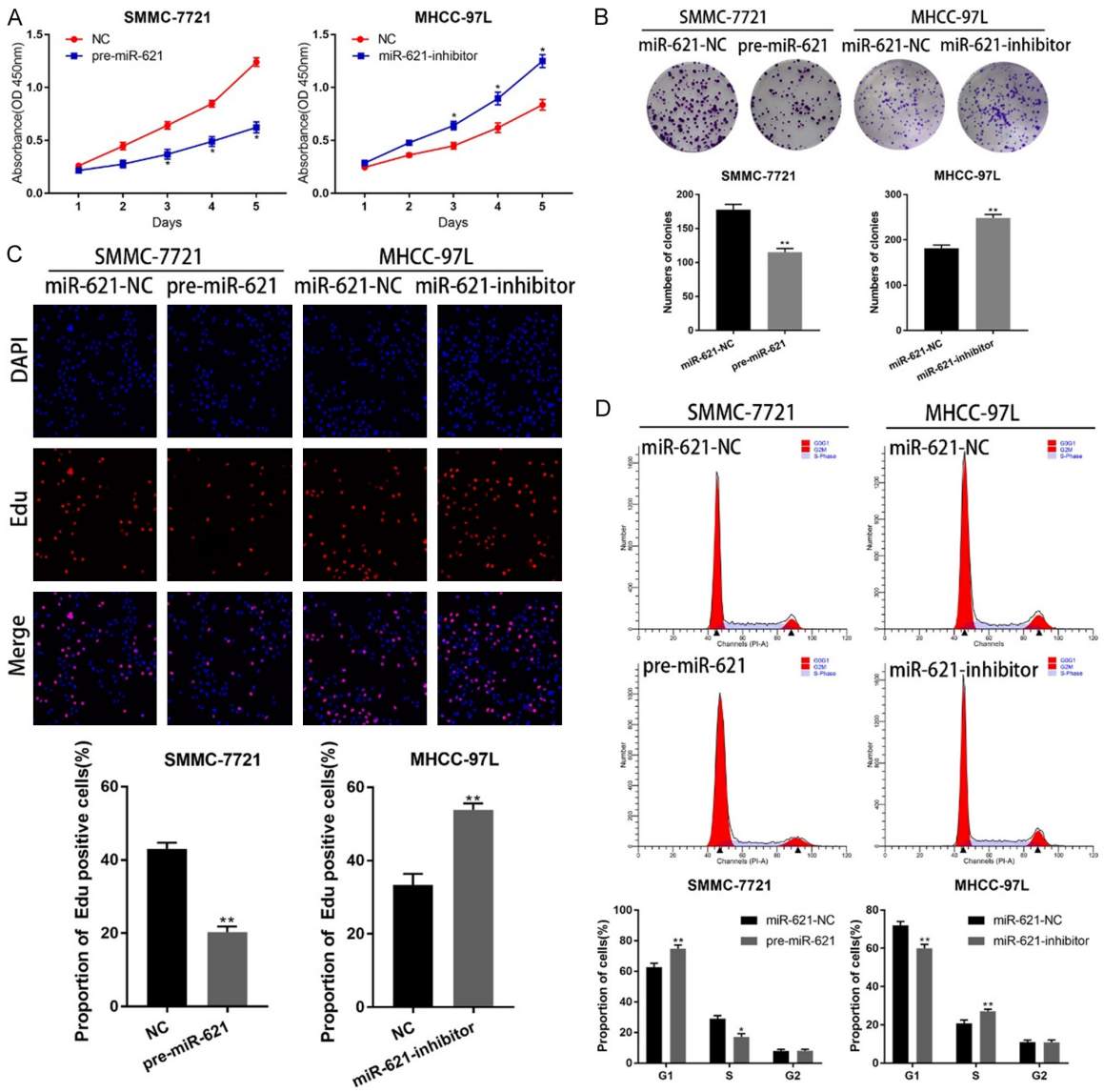

Figure 2. miR-621 suppresses HCC cell proliferation and induces cell cycle arrest. A. CCK-8 assays in the SMMC-7721 and MHCC-97L cells overexpressing or silencing miR-621. B. Colony formation assays in the SMMC-7721 and MHCC-97L cells overexpressing or silencing miR-621. C. 5-ethynyl-2'-deoxyuridine (EdU) incorporation assays were performed to assess the cell proliferation in the miR-621 overexpressing or silencing SMMC-7721 and MHCC-97L cells. The EdU-positive cells were counted from three random microscopic fields for each well. D. The flow cytometry results showing the cell cycle distribution of the miR-621 overexpressing or silencing SMMC-7721 and MHCC-97L cells. All data are presented as the mean ± S.E.M. (*P < 0.05, **P < 0.01, and ***P < 0.001).

621 can regulate CCND2/c-MYC by targeting CAPRIN1.

Xenograft nude mouse model of in vivo tumorigenesis

To further examine the contribution of miR-621 *in vivo*, SMMC-7221 and MHCC-97L cells transfected with lentivirus as previously described were injected into nude mice. Tumor growth was recorded every 3 days, and the mice were euthanized 30 days later. The tumors generated from the SMMC-7721-pre-miR-621 group were significantly smaller and lighter than the NC group, whereas the MHCC-97L group showed the opposite trend (**Figure 6A-C**).

In addition, we evaluated miR-621 expression in xenografts by qRT-PCR. The SMMC-7721 group transfected with pre-miR-621 showed high miR-621 expression levels, whereas the MHCC-97L group transfected with the miR-621

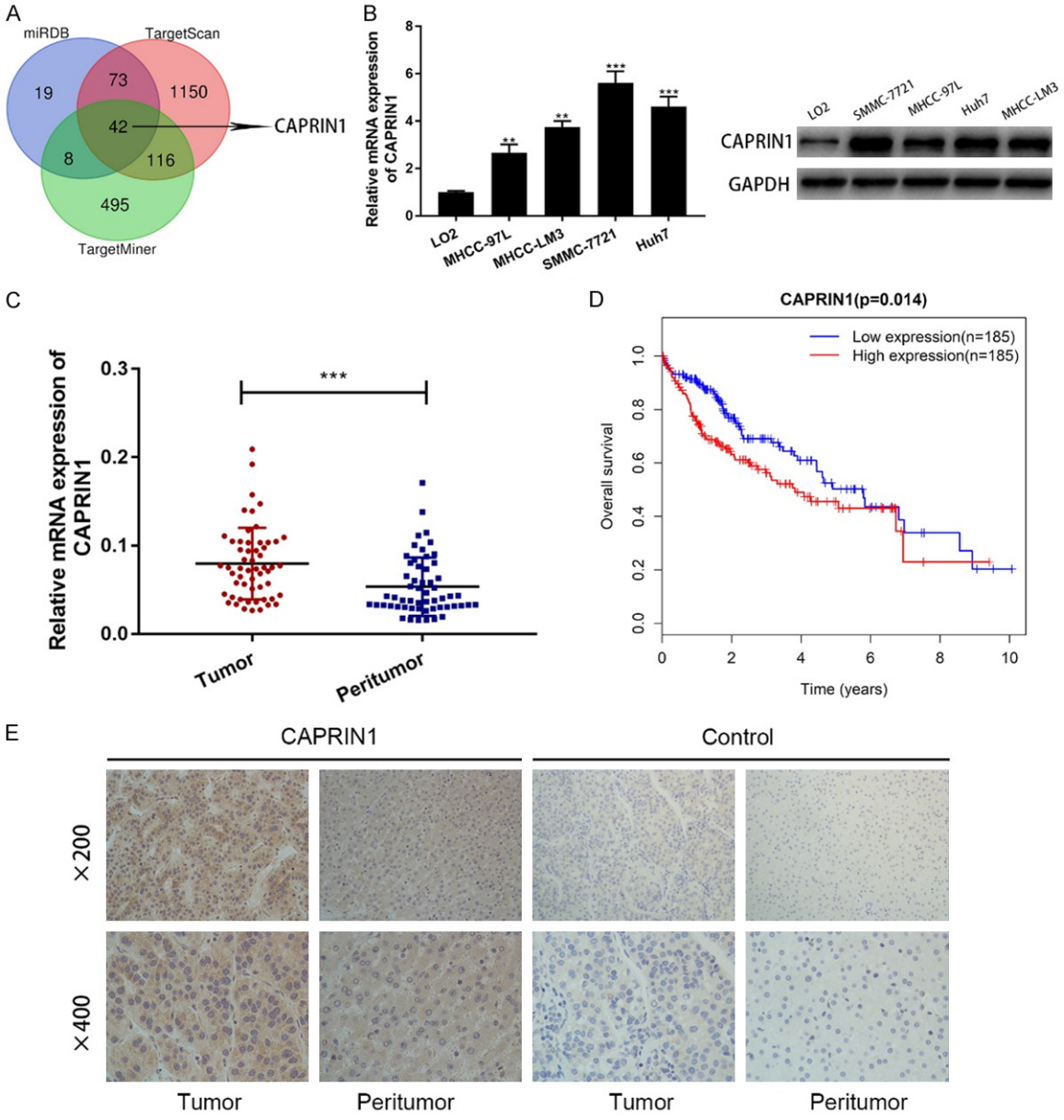

Figure 3. CAPRIN1 is upregulated in human HCC tissues and cell lines. A. Venn diagram displaying miR-621 computationally predicted to target CAPRIN1 by three different prediction algorithms: TargetScan, miRDB, and TargetMiner. B, C. Expression levels of CAPRIN1 in the HCC tissues and cell lines were detected by qRT-PCR and western blot. D. Kaplan-Meier analysis for overall survival of patients with HCC according to CAPRIN1 expression level in all patients (TCGA datasets). E. Representative immunostaining images of CAPRIN1 in HCC tissues and the corresponding peritumor samples. Immunostaining images using secondary antibody alone are shown as the control. All data are presented as the mean ± S.E.M. (*P < 0.05, **P < 0.01, and ***P < 0.001).

inhibitor presented lower expression levels (**Figure 6D** and **6E**). Then, western blotting and immunohistochemistry were conducted to examine the expression level of CAPRIN1 in xenografts. CAPRIN1 was significantly downregulated in the SMMC-7721-pre-miR-621 group and upregulated in the MHCC-97L-miR-621-inhibitor group. We also stained the xenografts with Ki-67. As shown in **Figure 6F**, the results further confirmed the suppressor role of miR-621 in HCC cell proliferation. Taken together, our data suggested that miR-621 suppressed HCC tumorigenesis by inhibiting CAPRIN1 expression.

Discussion

Due to the lack of effective therapies, HCC is still one of the leading causes of tumor-related

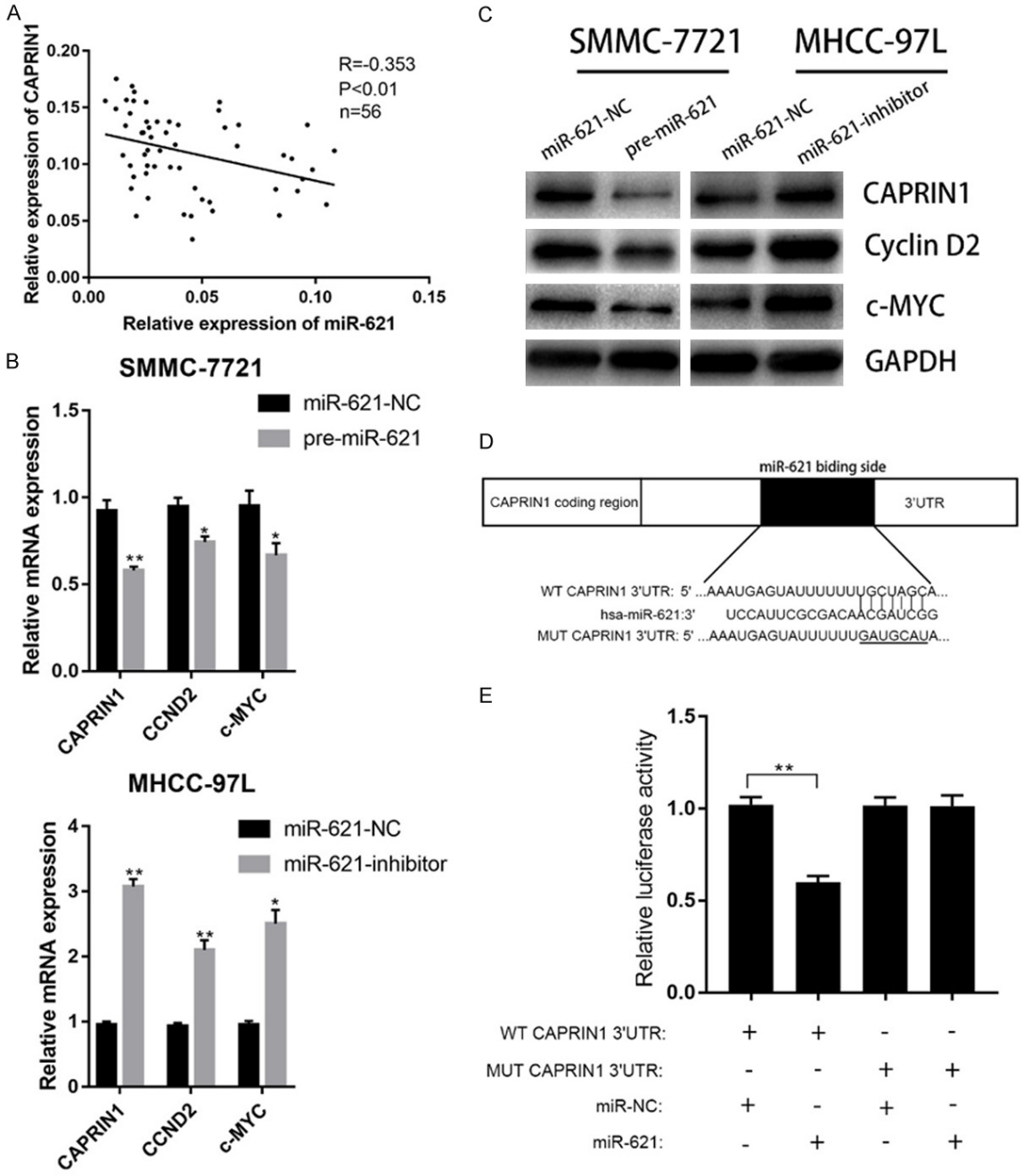

Figure 4. miR-621 directly targets CAPRIN1 in HCC cells. A. Spearman correlation analysis was conducted to confirm the correlations between the CAPRIN1 mRNA and miR-621 expression levels in the 56 HCC samples (r = -0.353, P < 0.01). B. The expression levels of CAPRIN1, CCND2, and c-MYC in the SMMC-7721 cells transfected with pre-miR-621 or miR-621-NC and the MHCC-97L cells transfected with miR-621-inhibitor or miR-621-NC were detected by qRT-PCR. C. Western blotting analysis of the expression levels of CAPRIN1, CCND2, and c-MYC in the SMMC-7721 cells transfected with pre-miR-621 or miR-621-NC and the MHCC-97L cells transfected with the miR-621-inhibitor or miR-621-NC. D. The predicted miR-621 targeting sequence in the CAPRIN1 3'-UTR (WT CAPRIN1 3' UTR). The target sequences of the CAPRIN1 3' UTR were mutated (MUT CAPRIN1 3' UTR). E. Dual-luciferase reporter assay of the cells transfected with WT CAPRIN1 3' UTR or MUT CAPRIN1 3' UTR reported together with 40 nM of the miR-621 mimic or negative control oligoribonucleotides. All data are presented as the mean ± S.E.M. (*P < 0.05, **P < 0.01, and ***P < 0.001).

death globally [1]. Increasing evidence has demonstrated that miRNAs play vital roles in the pathogenesis of HCC by functioning as oncogenes or tumor suppressor genes [22-24].

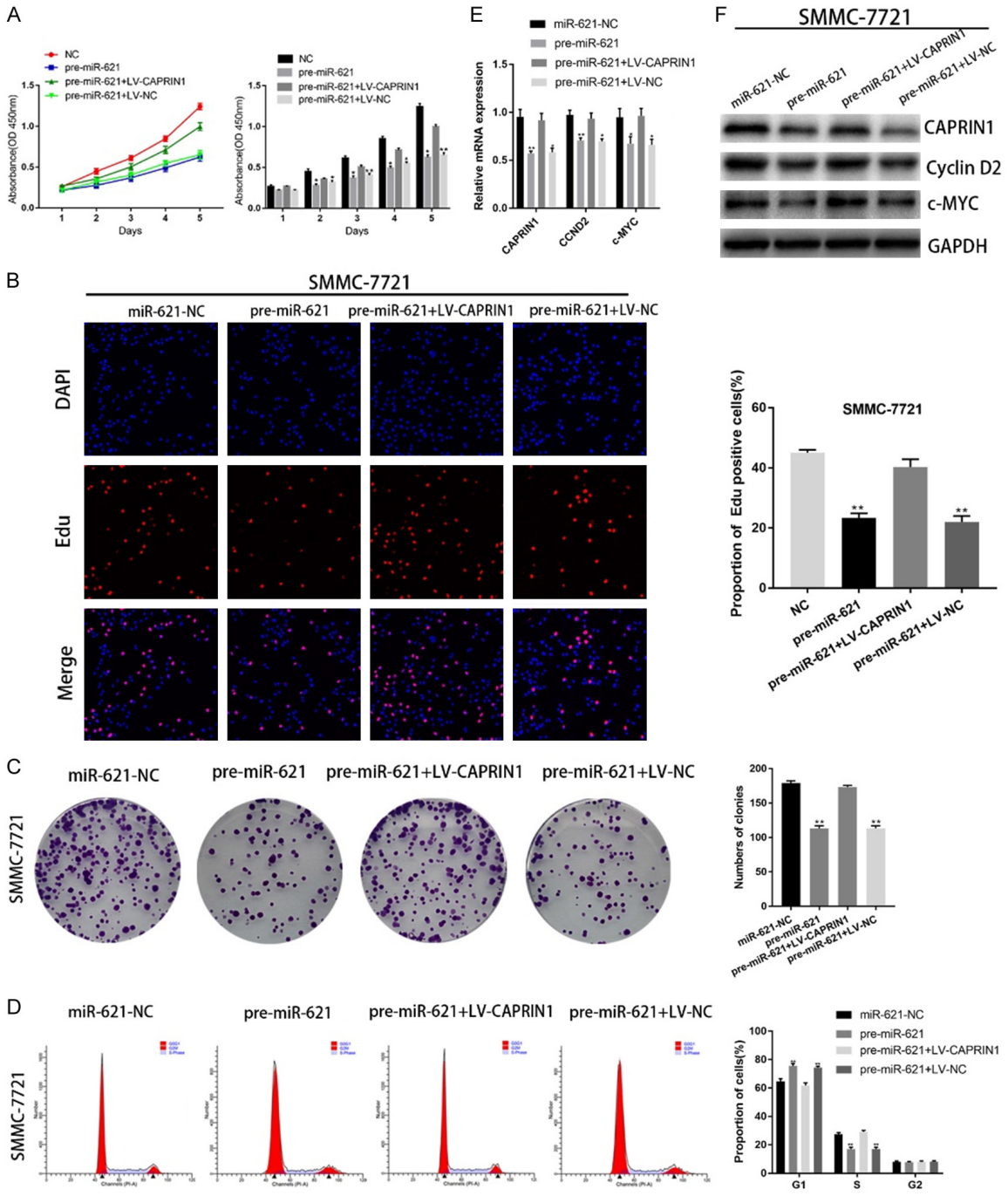

Figure 5. Rescue experiments were performed to confirm that CAPRIN1 is the functional target of miR-621 associated with HCC progression. A. CCK-8 analysis of the SMMC-7721-pre-miR-621 cells transfected with LV-CAPRIN1 or LV-NC. B. The 5-ethynyl-2'-deoxyuridine (EdU) incorporation analysis of miR-NC, pre-miR-621, pre-miR-621 plus LV-CAPRIN1, and pre-miR-621 plus LV-NC in the SMMC-7721 cells. The EdU-positive cells were counted from three random microscopic fields for each well. C. Colony formation ability of the SMMC-7721-pre-miR-621 cells transfected with LV-CAPRIN1 or LV-NC. D. Cell cycle analysis of the SMMC-7721-pre-miR-621 cells transfected with LV-CAPRIN1 or LV-NC. E, F. The expression of CAPRIN1 together with its downstream partners, CCND2 and c-MYC, at the mRNA and protein level in the SMMC-7721 cells transfected with the miR-621 overexpression lentivirus (iR-621) and CAPRIN1 overexpression lentivirus (LV-CAPRIN1). All data are presented as the mean ± S.E.M. (*P < 0.05, **P < 0.01, and ***P < 0.001).

Moreover, miRNA-based therapies have been used in several preclinical models, including liver cancer [25]. Therefore, investigation of miRNAs as contributors to the pathogenesis of

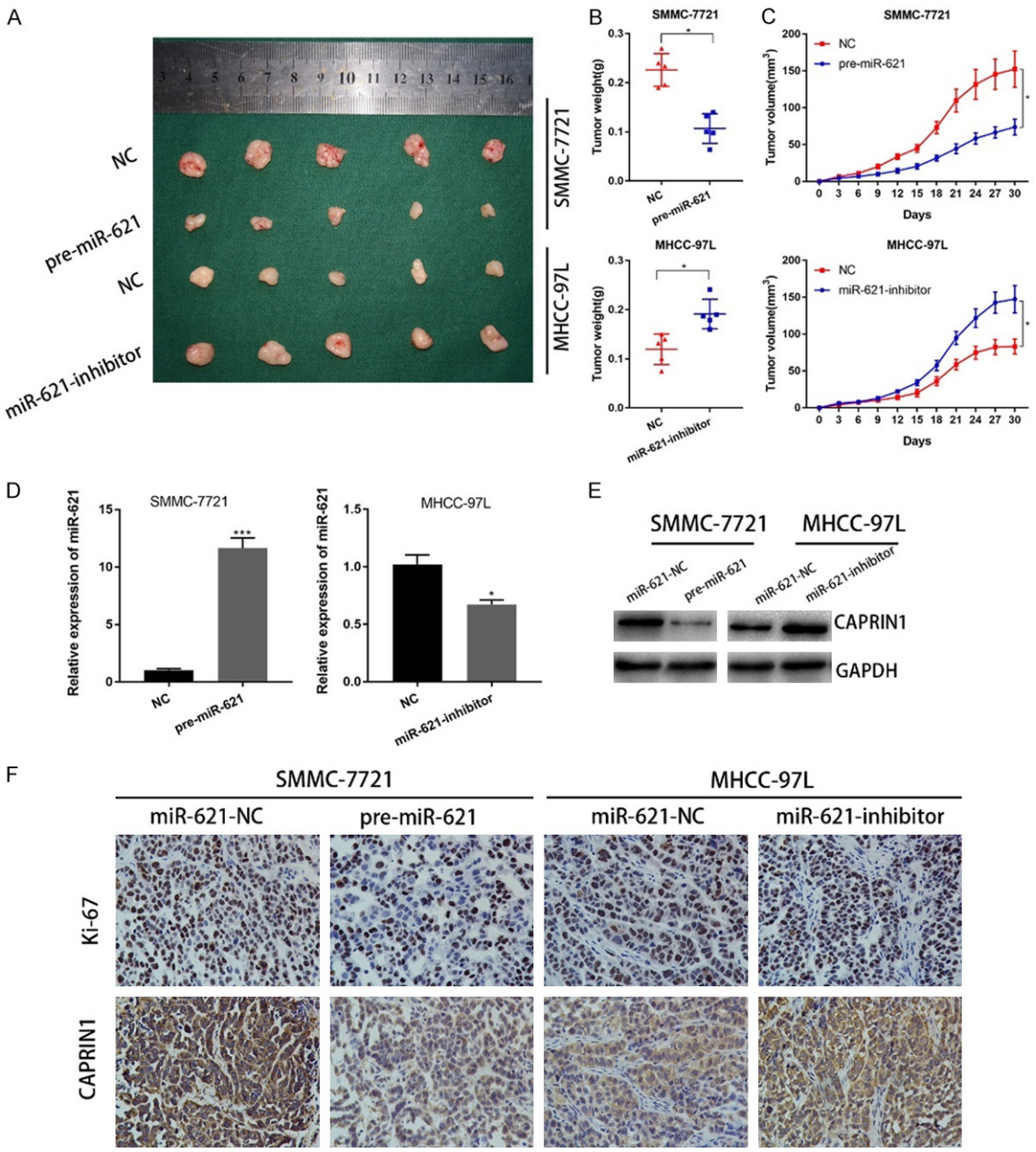

Figure 6. miR-621 suppresses xenograft tumor growth in *vivo*. A-C. Photographs of tumors derived from the different groups of nude mice transfected with pre-miR-621, miR-621-inhibitor, and miR-NC. The tumors were measured for volume and average weight. D. The miR-621 expression levels of xenografts were analyzed by qRT-PCR. E. The CAPRIN1 expression levels of xenografts were detected by western blot. F. Representative immunostaining images measuring the CAPRIN1 and Ki-67 expression levels in tumors derived from mice. All data are presented as the mean ± S.E.M. (*P < 0.05, **P < 0.01, and ***P < 0.001).

HCC will greatly promote the development of strategies for HCC diagnosis, prognosis, and treatment.

miR-621, together with the tumor suppressor genes miR-16-1 and miR-15a, is located on chromosome 13q. Skawran et al. inferred that the loss of miR-621, miR-16-1 and miR-15a due to chromosome 13q deletion might result in the upregulation of genes involved in cycle regulation, leading to uncontrolled cell growth in dedifferentiated HCC [16]. Previous studies have demonstrated the suppressor role of miR-16-1 and miR-15a in various tumors [26-29].

Recent research has revealed miR-621 as a tumor suppressor gene in breast cancer. Nevertheless, the functions and molecular mechanisms of miR-621 in HCC cells remain unknown. Our research demonstrated the downregulation of miR-621 in HCC tissues and cell lines and that a low expression level of miR-621 indicated a large tumor size in HCC patients. Further investigation revealed the function of miR-621 in cell proliferation and cell cycle distribution both *in vivo* and *in vitro*. We found that upregulated miR-621 suppressed cell growth and induced G0/G1 cell cycle arrest, whereas knockdown of miR-621 resulted in a marked increase in proliferation and in the population of cells in S phase of the cell cycle. Taken together, our data suggest that miR-621 functions as a tumor suppressor whose downregulation may lead to the progression of HCC.

In the subsequent mechanistic study, we first predicted the downstream target gene of miR-621 by biological tools. CAPRIN1 was selected as a potential target of miR-621 according to its biological function in tumors [30-32]. CAPRIN1 belongs to a highly conserved protein family throughout vertebrate evolution. CAPRIN1 is closely associated with cancer cell cycle and cell proliferation. Wang et al. demonstrated that CAPRIN1 was essential for normal cellular proliferation [31]. Grill et al. found that suppression of CAPRIN1 inhibited cell proliferation through prolongation of the G1 phase of the cell cycle [32]. In this study, we demonstrated that CAPRIN1 expression was upregulated and negatively correlated with miR-621 expression in HCC tissues. Then, the luciferase activity assay was conducted to validate the targeting of miR-621 to the 3' UTRs of CAPRIN1. Furthermore, we found that restoration of CAPRIN1 partially reversed the miR-621-mediated proliferation-inhibiting effect and cell cycle arrest in HCC cells. These data suggested that miR-621 suppressed HCC cell proliferation and induced G0/G1 cell cycle arrest by targeting CAPRIN1. Interestingly, Drougat L et al. reported that CAPRIN1 directly binds to the mRNAs for CCND2 and c-MYC through its carboxy-terminal RGG-rich region [20]. The c-MYC oncogene exerts a vital role in the genesis of many human cancers [33]. CCND2 can inhibit cell proliferation by inducing G0/G1 arrest [34, 35]. By western blot and qRT-PCR, we found decreased expression of c-MYC and CCND2 in the pre-miR-621 group, whereas the miR-621-inhibitor group showed the opposite trend. Further rescue experiments confirmed that the expression changes of c-MYC and CCND2 caused by dysregulation of miR-621 could be partially reversed by re-expression of CAPRIN1.

Nevertheless, there are some limitations in this research. First, owing to the small sample size of HCC tissues and cell lines, further research should be conducted to confirm the biological function of miR-621. Second, although our present study revealed the relationship between miR-621 and CAPRIN1, it is anticipated that other target genes of miR-621 may also participate in the biological function of miR-621. The underlying mechanisms require further investigation.

Conclusions

In summary, our findings suggest that miR-621 functions as a tumor suppressor in HCC and might contribute to HCC progression. Higher levels of miR-621 in HCC cells induced G0/G1 cell cycle arrest and cell proliferation repression via reduction of CAPRIN1 expression, indicating that miR-621 may serve as a promising biomarker for diagnosis and prognosis and could be a potential target for anti-HCC therapeutic applications.

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Disclosure of conflict of interest

None.

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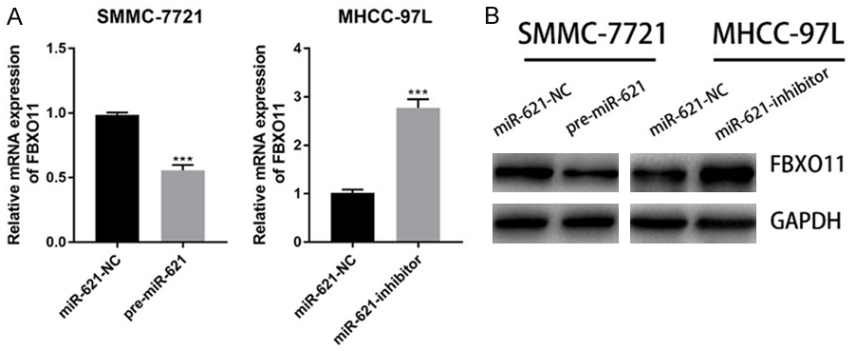

Figure S1. FBXO11 was taken as positive control to show the efficacy of pre-miR-621 and miR-621-inhibitor. A, B. The SMMC-7721 and MHCC-97L cells were transfected with lentivirus overexpressing miR-621 (defined as pre-miR-621) or lentivirus with short hairpin RNA targeting miR-621 (defined as miR-621-inhibitor). The negative control (NC) cells included a nontargeting sequence. FBXO11 expression levels were analyzed by qRT-PCR and western blot. All data are presented as the mean ± S.E.M. (*P < 0.05, **P < 0.01, and ***P < 0.001).

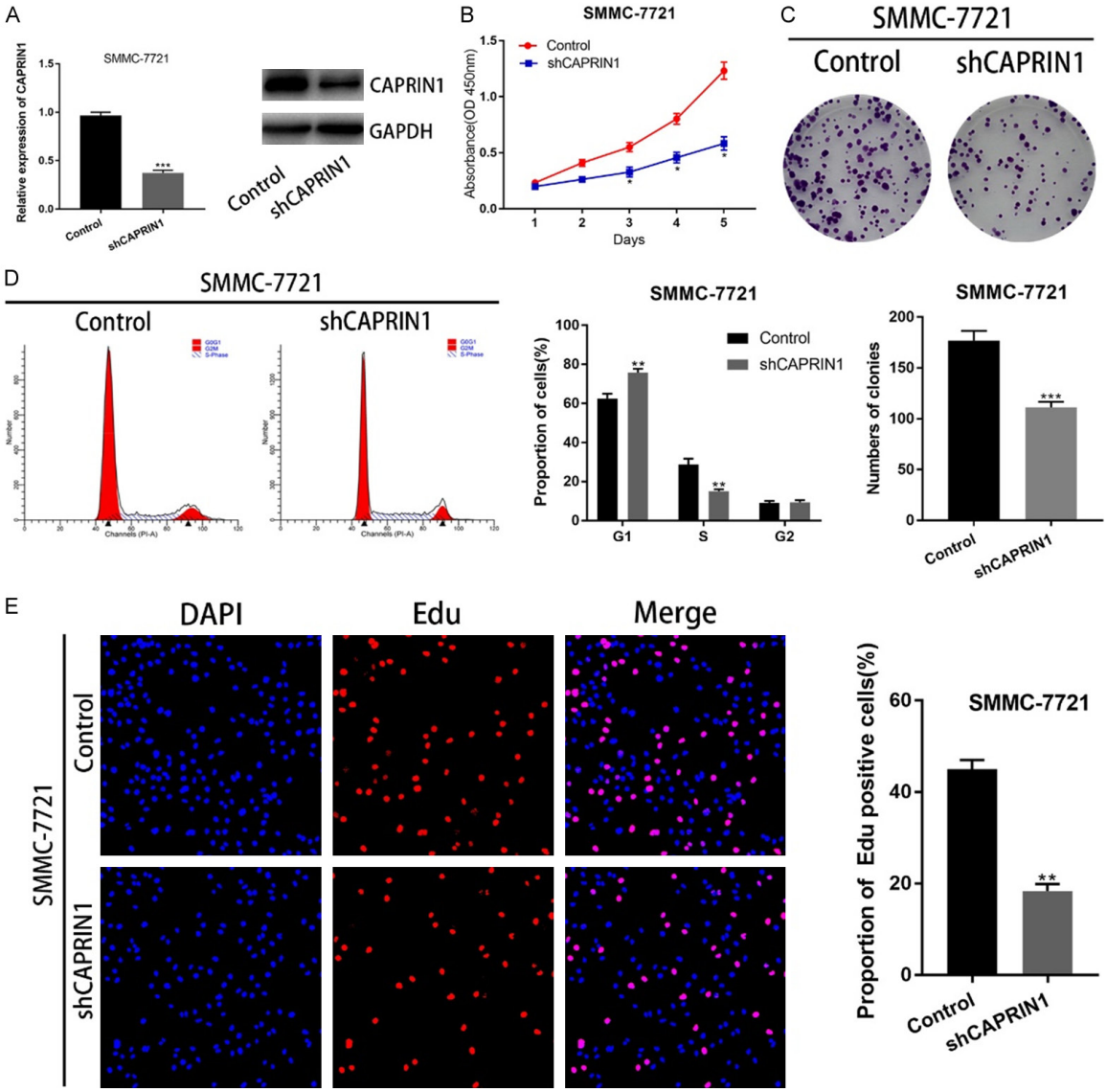

Figure S2. CAPRIN1 knocking down suppresses HCC cell proliferation and induces cell cycle arrest *in vitro*. A. The SMMC-7721 cells were transfected with lentivirus knocking down CAPRIN1 (defined as shCAPRIN1). The efficacy of shCAPRIN1 was analyzed by qRT-PCR and western blot. B. CCK-8 assays in the SMMC-7721 cells knocking down CAPRIN1. C. Colony formation assays in the SMMC-7721 cells knocking down CAPRIN1. D. The flow cytometry results showing the cell cycle distribution of the CAPRIN1 knocking down SMMC-7721 cells. E. 5-ethynyl-2'-deoxyuridine (EdU) incorporation assays in the SMMC-7721 cells knocking down CAPRIN1. The EdU-positive cells were counted from three random microscopic fields for each well. All data are presented as the mean ± S.E.M. (*P < 0.05, **P < 0.01, and ***P < 0.001).

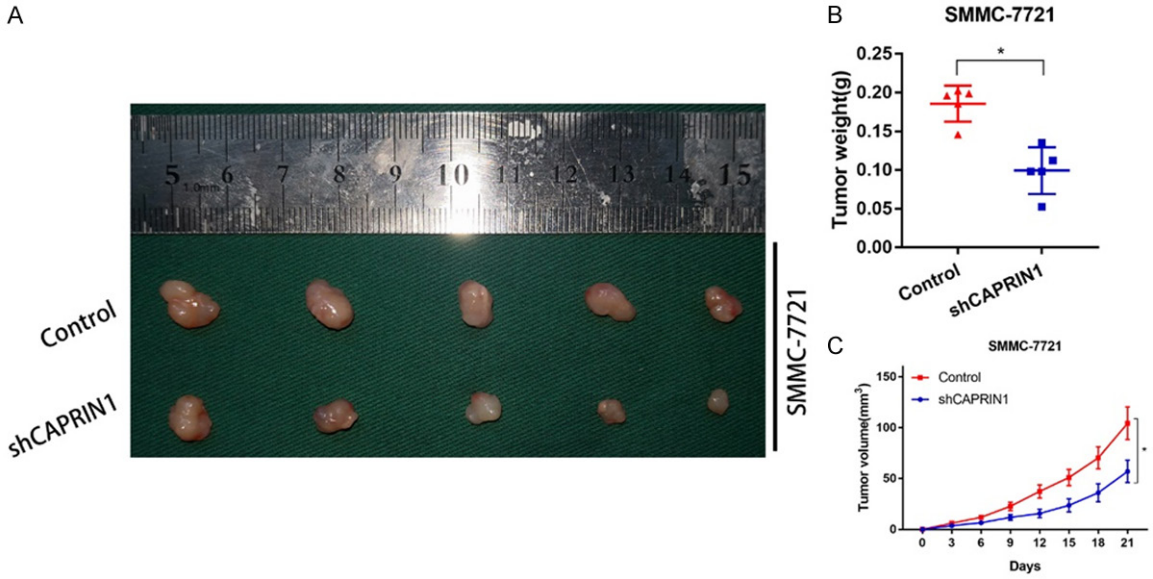

Figure S3. CAPRIN1 knocking down suppresses xenograft tumor growth. A. Photographs of tumors derived from the different groups of nude mice transfected with shCAPRIN1 and sh-NC. B, C. The tumors were measured for volume and average weight. All data are presented as the mean ± S.E.M. (*P < 0.05, **P < 0.01, and ***P < 0.001).