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# MiR-637 inhibits proliferation and invasion of hepatoma cells by targeted degradation of AKT1

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**Abstract. – OBJECTIVE: This study aimed at investigating whether miR-637 could promote proliferation of hepatocarcinoma cells by targeted regulation of AKT1 expression, leading to enhanced cell invasion and thus participating in the progression of liver cancer.**

**PATIENTS AND METHODS: The miR-637 and AKT1 expressions in cancer tissues and adjacent tissues of liver cancer patients were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The effects of miR-637 on cell proliferation and cell invasion were examined by cell counting kit-8 (CCK-8) and cell invasion assays. Dual-luciferase reporter gene assay was performed to evaluate the regulating relationship between miR-637 and AKT1. Also, the expression of AKT1 after overexpression or knockdown of miR-637 was analyzed by Western blot to evaluate whether miR-637 could affect proliferative and invasive ability of hepatoma cells by inhibiting the expression of AKT1.**

**RESULTS: The qRT-PCR results revealed that miR-637 expression in cancer tissues of liver cancer patients was markedly lower than that in corresponding adjacent tissues, which was consistent with its low expression in HCC cell lines. However, AKT1 expression in cancer tissues was markedly higher than that in corresponding adjacent tissues. Overexpression of miR-637 in hepatoma cells inhibited cell proliferation and attenuated cell invasion, while inhibition of miR-637 showed the opposite effect. Results of dual-luciferase reporting assay and Western blot demonstrated that miR-637 can selectively degrade AKT1 and that overexpression of AKT1 in HCC cells can partially reverse the effect of miR-637 on cell proliferation and invasion.**

**CONCLUSIONS: MiR-637 can promote the proliferation of hepatoma cells and enhance the cell invasive ability, the mechanism of which may be related to the targeted regulation of AKT1 expression.**

*Key Words:*

MiR-637, Liver cancer, Cell proliferation, Cell invasion, AKT1.

## Introduction

Liver cancer is one of the most common malignant tumors in the world, and more than 90% of them are hepatocellular carcinoma[1]. Liver cancers are characterized by rapid proliferation and invasion, which are the important factors leading to the poor prognosis of many liver cancer patients and low 5-year survival rate[2,3]. Therefore, revealing the molecular mechanism of rapid cell proliferation and invasion is of great importance for improving the prognosis of liver cancer patients.

The PI3K/Akt signaling pathway plays a vital role in cell proliferation, cell cycle, and apoptosis of hepatocellular carcinoma, ovarian cancer, melanoma, and other malignant tumors[4-6]. Scholars[7,8] have shown that the main activation process of the P13K/Akt pathway is that the domain with a phosphorylation site binds to the phospholipid molecule phosphatidylinositol 3 phosphate (PIP3) and recruits the P13K subunit p85 to the vicinity of cell membrane, which further activates the proliferation and apoptosis-related genes so as to alter the cell cycle and regulate the biological behavior of tumor cells. AKT1 is the upstream protein in the P13K/Akt signaling pathway, and its phosphorylation process activates this pathway and regulates tumor cell proliferation and apoptosis[9]. Studies have shown that PI3K/Akt signaling pathway can further accelerate the infiltration and migration of tumor cells by enhancing the proliferative ability of tumor cells; meanwhile, this pathway may affect levels of some proteins regulating tumor cell apoptosis so as to promote the activation or inhibition of endogenous apoptotic genes[10].

MicroRNAs (miRNAs) are a series of small non-coding single-stranded RNAs ranging from 18 to 25 nt that bind to target mRNA by partial complementation at the post-transcriptional level,

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leading to mRNA degradation and translational inhibition[11]. Many miRNAs inhibit the transcription of tumor-related genes through targeted regulation of downstream key molecules, thereby inhibiting tumor proliferation, invasion, migration, and chemoradiation resistance. It has been found that the dysregulation of certain miRNAs plays a key role in the development of tumors[12]. In addition, several experimental results indicate that miRNAs have potential value to become candidate markers and therapeutic targets for cancer[13]. MiR-637 has been confirmed to be essential in the pathogenesis of cancers such as papillary thyroid carcinoma, pancreatic ductal adenocarcinoma, and melanoma[14-16]. Previous studies have demonstrated that miR-637 is lowly expressed in liver cancer tissues and cell lines[17]; however, its specific mechanism involved in occurrence or progression of liver cancer remains unclear.

The aim of this study was to explore the effects of miRNA-637 on the proliferative and invasive capacity of hepatoma cells and to verify whether miR-637 can exert its biological functions by targeting AKT1 expression.

## Patients and Methods

### Study Subjects and Sample Collection

46 cases of liver cancer tissues and corresponding adjacent tissues were derived from specimens of patients with liver cancer after radical surgery. Fresh tissues obtained from the lesion were taken by a special clamp and immediately placed in a liquid nitrogen tank for cryopreservation. Patient-related clinical data were then collected. All of the above tissue samples were collected with the patient's consent. And this study was approved by the Ethics Committee of Huai'an First People's Hospital.

### Cell Culture and Transfection

The normal cell line THLE-2 and the liver cancer cell lines including SMMC-7721, HepG2, Hep3B, Huh-7, and Sk-Hep-1 were all purchased from Shanghai ATCC (American Type Culture Collection) Company (Shanghai, China). Cells were cultured in RPMI (Roswell Park Memorial Institute) or DMEM (Dulbecco's Modified Eagle Medium) medium (HyClone, South Logan, UT, USA) containing 10% inactivated newborn bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin in a 37°C incubator with 90% relative humidity and 5% $CO_2$. Cells reaching 50%-60% of confluency were used for transfection by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The transfection effect was then verified by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) assay. The miRNA mimics, inhibitor, pcDNA-AKT1 and the corresponding negative control (pcDNA-NC) used in this experiment were all provided by Gene Pharma (Shanghai, China).

### RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Detection

Total RNA of cells or tissues was extracted using a TRIzol kit (Invitrogen, Carlsbad, CA, USA), and a reverse transcription reaction system was prepared on ice using a PrimeScript RT reagent Kit (TaKaRa, Code No. RR037A, Otsu, Shiga, Japan). Quantitative PCR operations were performed according to the SYBR Green PCR Kit instructions, with 10 μL of the total reaction system. The primer were listed below: miR-637 (F: 5'-ACUGGGGGCUUUCGGGCUCUGCGU-3' R: 5'-ACGCAGAGCCCGAAAGCCCCCAGU-3'), U6 (F: 5'-CGCTTCGGCAGCACATATTAC-3' R: 5'- TTCACGAATTTGCGTGTCCAT-3'), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (F: 5'-GGAATCCACTGGCGTCTTCA-3' R: 5'-GGTTCACGCCCATCACAAAC-3'), AKT1 (F: 5'-CTGAGATTGTGTCAGCCCTGGA-3' R: 5'-CACAGCCCGAAGTCTGTGATCTTA-3'.

### Cell Counting Kit-8 (CCK-8) Assay

24 h after transfection, cells were digested and seeded into 96-well plates at $2*10^3$ per well, with 6 replicate wells set up. The viability of the cells was determined at 6 h, 24 h, 48 h, 72 h, and 96 h after seeding, respectively. 2 h before the test, 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added, and then, the cells were placed at 37°C for 1 h. The absorbance at 450 nm of each well was measured using a microplate reader.

### Cell Invasion Assay

Matrigel was diluted to 1 mg/mL with serum-free medium pre-cooled at 4°C and then coated on the upper chamber of transwell and placed 3-5 h at 37°C. The cells in logarithmic growth phase were digested, suspended and adjusted to $2\times10^5$ cells/mL. 600 μL of medium were added in the lower chamber while 100 cell suspensions were added in the upper chamber. Cells were further cultured for 24 hours at 37°C in the incubator. Afterwards, cells in the upper chamber

were fixed in the formaldehyde for 30 minutes and stained by crystal violet for 15-30 minutes. At last, results of cell invasion were observed using a microscope.

### Western Blot Assay

Cells were lysed with cell lysate containing protease inhibitor phenylmethylsulfonyl fluoride (PMSF) and then scraped off to obtain total proteins (Beyotime, Shanghai, China). The lysate was aspirated and centrifuged at 12 000 r/min for 20 min. The total protein concentration was determined by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). 50 μg of total protein from each sample were applied to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel for electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocked for 2 hours with skim milk, the proteins in the membrane were incubated with the specific primary antibody separately overnight. In the next day, the protein bands were incubated with the secondary antibody and visualized using enhanced chemiluminescence (ECL) luminescence (Thermo Fisher Scientific, Waltham, MA, USA).

### Dual-Luciferase Reporter Gene Assay

Binding sites of miR-637 and AKT1 were predicted by Target Scan to construct wild-type and mutant plasmids in Ruizhen (Shanghai, China). MiR-637 mimics and negative controls were subsequently synthesized at Gena Pharma (Shanghai, China). The relative dual-luciferase values were detected by a standardized method after plasmids transfection. The same experiment was repeated 3 times and the average value was calculated.

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM, Armonk, NY, USA) was used for data analysis. Measurement data were analyzed by the *t*-test and expressed as mean ± standard deviation. The difference was statistically significant at $p < 0.05$.

## Results

### MiR-637 Has a Low Expression in Liver Cancer Tissues

MiR-637 and AKT1 expression were detected in 46 cases of HCC and corresponding adjacent tissues using quantitative PCR assay. Results showed that miR-637 was lowly expressed in liver cancer tissues (Figure 1A), while AKT1 was highly expressed in those tissues (Figure 1B). Moreover, it was found that the overall survival time of patients in the miR-637 high expression group was higher than those in the low expression one (Figure 1C). At the same time, miR-637 was found to have a good sensitivity and specificity in the diagnosis of liver cancer by plotting the ROC curve (Figure 1D). These results showed that miR-637 might be used as a biomarker for early diagnosis of liver cancer.

### MiR-637 Promotes Proliferation and Invasion of Liver Cancer Cells

MiR-637 was also found down-regulated in liver cancer cell lines including SMMC-7221, HepG2, Hep3B, Huh-7, and Sk-Hep-1 (Figure 2A), among which Huh-7 and Sk-Hep-1 showed the most pronounced difference. The two cells were then selected for subsequent experiments. Then, miR-637 mimics, a miR-637 inhibitor as well as their negative controls were transfected into cells respectively to investigate the effect of miR-637 on cell proliferation and invasion. Transfection efficiency was examined and shown (Figure 2B, 2C). CCK8 results indicated that overexpression of miR-637 inhibited cell proliferation, whereas the opposite result was observed by silencing miR-637 (Figure 2D). Subsequently, the result of the transwell assay revealed that upregulated miR-637 markedly weakened hepatocarcinoma cell invasion, while inhibition of miR-637 expression enhanced cell invasiveness (Figure 2E). The above analysis suggested that miR-637 could promote the proliferation and invasion of liver cancer cells.

### MiR-637 Can Selectively Degrade mRNA of AKT1

Next, we tried to find out the function mechanism of miR-637 on cell proliferation. We predicted the target gene of miR-637 by bioinformatics, performed functional analysis, and found that AKT1 might be the target of miR-637 (Figure 3A). The dual-luciferase reporting assay results showed that transfection of miR-637 mimics in Huh-7 and Sk-Hep-1 cells decreased the luciferase activity of AKT1-WT 3'UTR group (Figure 3B), indicating that AKT1 could bind to miR-637. We then analyzed miR-637 as well as AKT1 expression in HCC tissues and found a significant negative correlation between them

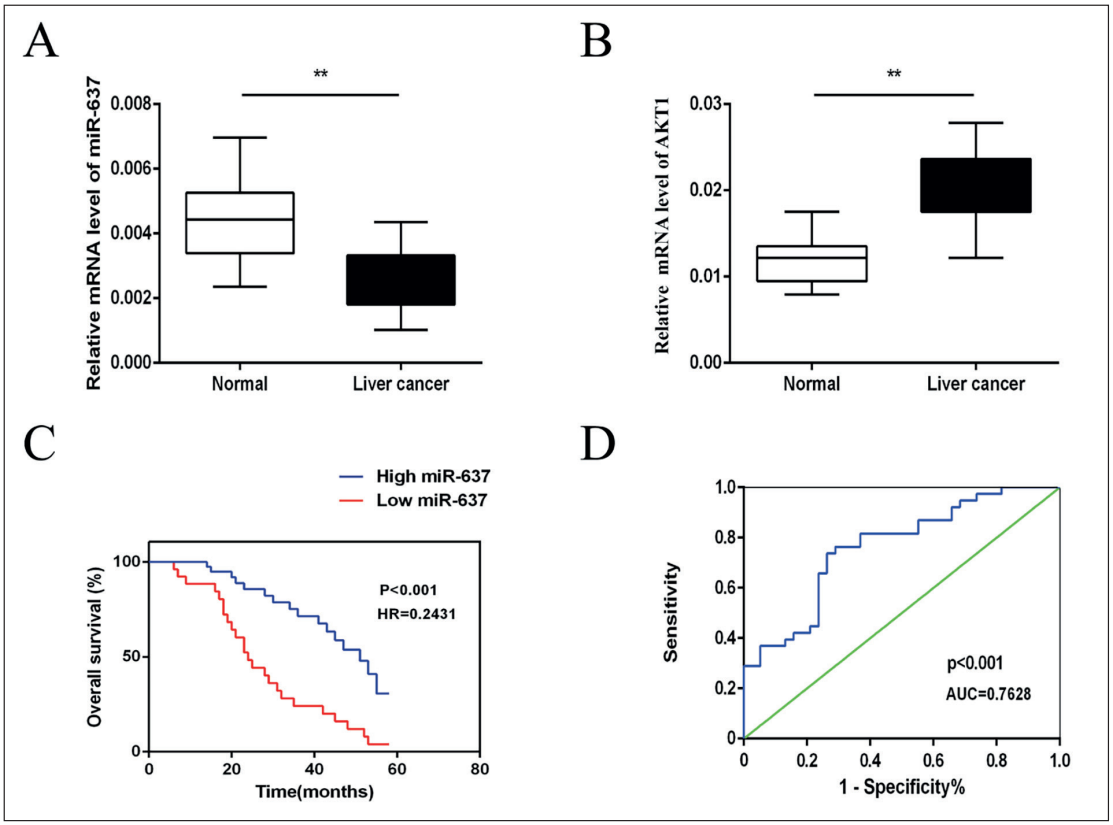


**Figure 1.** Expression levels and clinical features of miR-637 in liver cancer tissues. ***A,*** In 46 cases of HCC tissues and corresponding adjacent tissues, miR-637 expression was detected by qRT-PCR. ***B,*** AKT1 expression was detected by Qrt-PCR in liver cancer tissues in 46 cases of HCC and corresponding adjacent tissues. ***C,*** The survival time of miR-637 high expression group was higher than that of miR-637 low expression group. ***D,*** MiR-637 analysis of ROC curves in liver cancer tissues and adjacent tissues (AUC = 0.7628, $p < 0.001$).

(Figure 3C). In addition, we transfected cells with miR-637 mimics and miR-637 inhibitor to achieve miR-637 overexpression and knockdown. AKT1 mRNA expression was found decreased in miR-637 mimics group but elevated in miR-637 inhibitor group (Figure 3D). Meanwhile, Western blot analysis revealed the similar changes in the AKT1 protein levels (Figure 3E). Besides, mRNA along with protein expression of AKT1 was detected after transfection of pcDNA-AKT1, and it was found that pcDNA-AKT1 significantly increased AKT1 expression in cells (Figure 3F, 3G). These results demonstrated that miR-637 could bind to AKT1 selectively.

### *Overexpression of AKT1 Reverses the Effect of MiR-637 on Proliferation and Invasion*

After overexpression of miR-637 in cells, the CCK8 assay was performed to detect cell proliferation. It was found that cell proliferation was markedly attenuated but could be partially reversed after simultaneous overexpression of AKT1 (Figure 4A). Similarly, the AKT1 overexpression reversed the effect of up-regulated miR-637 on cell invasive capacity (Figure 4B). All the above results indicated that miR-637 may attenuate cell proliferation and cell invasion by inhibiting the expression of AKT1.

## Discussion

Liver cancer is one of the most common malignant tumors and the third leading cause of cancer-related death in the world. There are about 1 million of new cases of liver cancer every year, and the ratio of male to female is about 4:1[18]. In recent years, benefited from continuous improvement of comprehensive treatment, advancement of modern imaging technology and application of individualized treatment principles, the ther-

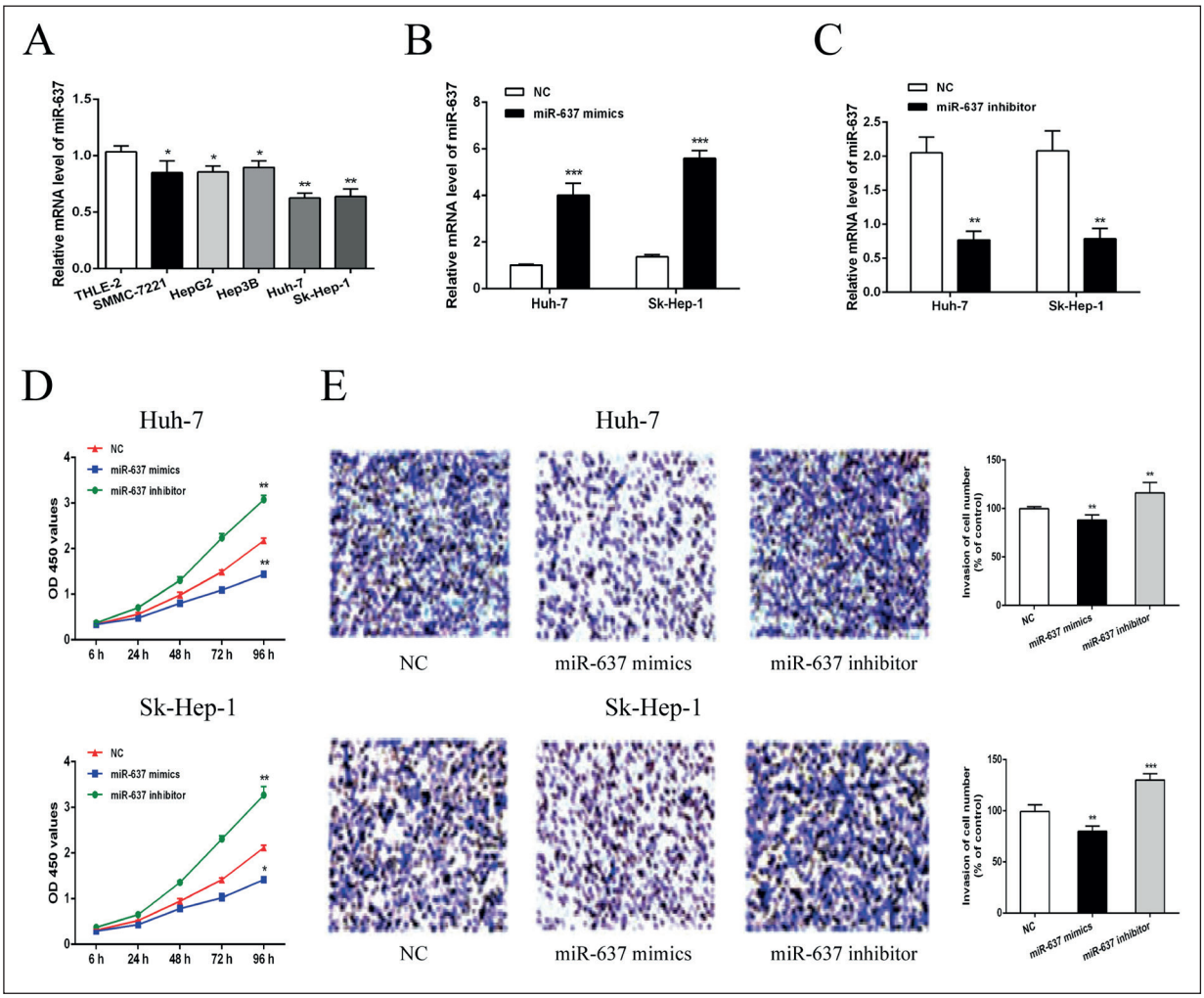


**Figure 2.** MiR-637 inhibits proliferation and invasion of liver cancer cells. ***A,*** Expression of miR-637 in normal cell lines and liver cancer cell lines. ***B,*** Transfection efficiency of miR-637 mimics in Huh-7 and Sk-Hep-1 cells. ***C,*** MiR-637 inhibitor transfection efficiency in Huh-7 and Sk-Hep-1 cells. ***D,*** In Huh-7 and Sk-Hep-1 cells, up-regulation of miR-637 expression inhibits cell proliferation and inhibits miR-637 expression, thereby promoting cell proliferation. ***E,*** In Huh-7 and Sk-Hep-1 cells, up-regulation of miR-637 expression inhibits cell invasion and inhibits miR-637 expression, and cell proliferation is enhanced.

apeutic effect of liver cancer has been greatly improved. However, the prognosis of liver cancer patients is still not satisfactory[19]. The main reason is that patients with liver cancer have been in an advanced stage at the time of diagnosis. Therefore, finding biomolecules capable of early diagnosis of liver cancer and more effective treatment methods is an urgent problem to be solved[20].

MiRNAs, a class of non-coding small RNAs of approximately 20-24 nucleotides in length, can regulate gene expression primarily through post-transcriptional regulation. Although miRNAs account for only 1-3% of human genes, they regulate more than 30% of gene expression[21]. Many studies have confirmed that miRNAs are involved in a variety of cellular functions. Its abnormal expression is associated with many diseases including tumors[22]. It is generally believed that miRNAs can exert tumor suppressing or promoting effect and a variety of miRNAs participate in the progression of liver cancer. For instance, miR-21 was found overexpressed in liver cancer tissues. Knockdown of it can inhibit tumor cell proliferation, migration, and invasion, while overexpressing it is capable of promoting tumor progression. Up-regulation of miR-21 can inhibit the expression of PTEN, thus affecting the progression of liver cancer. Meanwhile, the

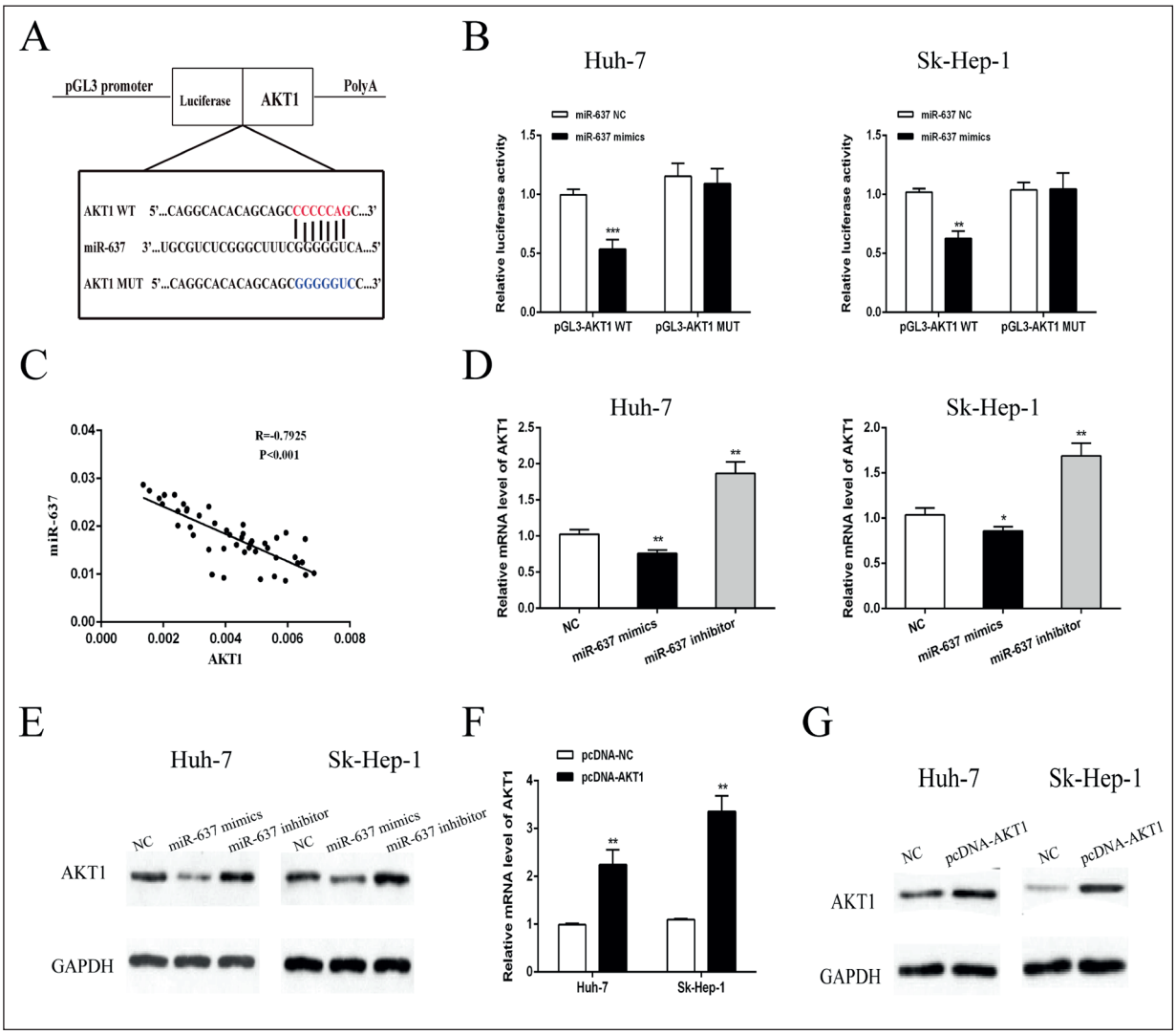


**Figure 3.** MiR-637 targets binding and degrades the expression of AKT1. ***A,*** The bioinformatics website predicts the binding site of miR-637 to AKT1. ***B,*** Dual-luciferase reporter gene results show that miR-637 can bind to the 3'-UTR of AKT1 and degrade AKT1. ***C,*** There was a significant negative correlation between miR-637 and AKT1 expression in cancer tissues and adjacent tissues. ***D,*** After up-regulating the expression of miR-637, the expression of AKT1 was significantly decreased in Huh-7 and Sk-Hep-1 cells. After inhibiting the expression of miR-637, the expression of the AKT1 was significantly increased. ***E,*** After up-regulating the expression of miR-637, the expression of AKT1 protein was significantly decreased in Huh-7 and Sk-Hep-1 cells, and the expression of the AKT1 protein was significantly increased after inhibiting the expression of miR-637. ***F,*** mRNA expression of AKT1 after transfection of pcDNA-AKT1 in Huh-7 and Sk-Hep-1 cells. ***G,*** Protein expression of AKT1 after transfection of pcDNA-AKT1 in Huh-7 and Sk-Hep-1 cells.

intervention of miR-21 expression can stimulate the occurrence and development of liver cancer through regulating PTEN-dependent pathway[23]. MiR-195 is down-regulated in hepatocarcinoma tissues, and up-regulation of its expression directly decreases levels of VEGF, VAV2, and CDC42, thereby inhibiting hepatic angiogenesis[24]. MiR-637 is one of the earlier discovered miRNAs, which is located on chromosome 19p13.3 and has autonomous transcription units. During tumorigenesis and development, miR-637 acts as a tumor suppressor gene. In our research, we focused on the function of miR-637 in liver cancer and explored its possible mechanism of affecting the biological function of liver tumor cells. It was shown that miR-637 expression was significantly reduced in tumor tissues of patients with liver cancer. Similar results were further verified in liver cell lines. These results suggested that miR-637 might be related to certain biological

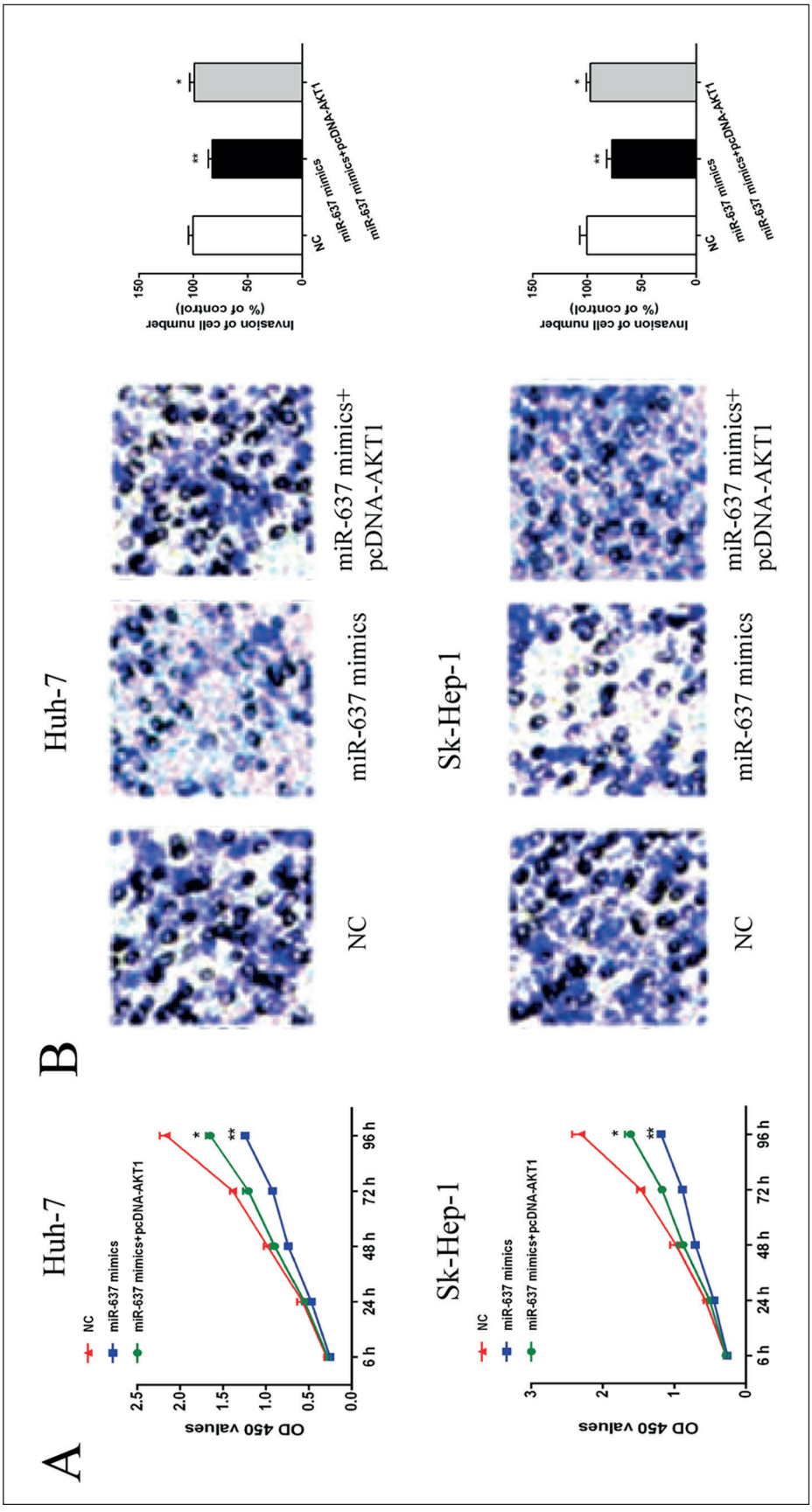


**Figure 4.** Overexpression of AKT1 reverses the effect of miR-637 on cell proliferation and invasion. ***A***, After overexpression of miR-637 in Huh-7 and Sk-Hep-1 cells, the cell proliferation ability was significantly attenuated, and the proliferation ability was enhanced after overexpression of AKT1. ***B***, Overexpression of miR-637, cell invasive ability is weakened, and at the same time overexpressing AKT1, cell invasive ability is enhanced.

functions of tumors. Based on the above studies, Sk-Hep-1 and Huh-7 cell lines were selected for functional studies of miR-637. The results showed that low expression of miR-637 promoted the proliferation and invasive ability of Huh-7 and Sk-Hep-1.

P13K/Akt signaling pathway regulates physiological functions such as cell proliferation and apoptosis[25], which can be activated by phosphorylation of Akt1. p85, as one of the important subunits of P13K, also plays an important part in this signaling pathway. Akt1 is involved in lots of cellular processes like cell proliferation, cell apoptosis, and glucose metabolism. Our study showed that AKT1 was highly expressed in liver cancer tissues and played a crucial role in the development of liver cancer. And it was found that inhibiting miR-637 could significantly promote tumor cell proliferation and enhance cell invasion. Further, bioinformatics was used to predict that AKT1 might be a downstream target gene of miR-637, which was then confirmed by luciferase reporter gene experiments. In addition, overexpression or inhibition of miR-637 could alter the expression of AKT1. Furthermore, it was verified that AKT1 complementation was able to restore the effect of miR-637 on hepatoma cells, suggesting that miR-637 may regulate the expression of AKT1 and thus affect the proliferation and invasion of liver cancer cells.

## Conclusions

We revealed that the miR-637 can target AKT1 so as to inhibit its expression. Low expression of miR-637 can promote the proliferation and invasion of hepatoma cells and thus participate in the progression of liver cancer. At the same time, it is especially encouraging that miR-637 may become a new biomarker for diagnosis of potential liver cancer.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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