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Silencing AKT1 inhibits proliferation of liver cancer cells and increases sensitivity to epirubicin

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Abstract: AKT serine/threonine kinase 1 (AKT1) overexpression is correlated with chemoresistance in liver cancer. However, the cellular functions and precise signals elicited by AKT1 in liver cancer cell lines and its relative chemoresistance have not been elucidated. In this study, we found that ATK1 was highly expressed in liver cancer cells and that the chemotherapeutic drug epirubicin (EPI) reduced the AKT1 expression. Then we used the transfected liver cancer cell lines SMMC-7721 and HepG2 with small interfering RNAs and the targeted vector to establish the AKT1 knockdown and overexpression cell lines. Our in vitro experiments showed that knocking down AKT1 in SMMC-7721 and HepG2 cells inhibited cell proliferation, induced cell apoptosis and cell cycle blocking, elevated the chemosensitivity of HepG2 cells to EPI, and upregulated the tumor suppressor gene phosphatase and tensin homolog (PTEN). Our findings may lead to a better understanding of the biological effect of AKT1 and may provide mechanistic insights on tumor development.

Keywords: liver cancer; AKT1; epirubicin (EPI); PTEN

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1. Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, accounting for 85% of liver cancer cases [1], and is the third most common cause of cancer-related deaths worldwide [2,3]. Patients with HCC generally present at an advanced stage due to compensated cirrhosis defined by the absence of pathognomonic symptoms, which results in death within 6–20 months [3,4,5]. Furthermore, the recurrence rate after curative resection is high at approximately 70% [6]. While surgery is the best treatment option for the early stages of HCC, as mo st HCC patients are diagnosed at later stages, chemotherapy is the mainstream treatment for these patients to slow tumor growth and reduce metastasis [7]. Although the novel chemotherapy regimen has improved the prognosis for HCC patients, chemoresistance has become the key barrier to the effective treatment of HCC with chemotherapeutic drugs and the cause of treatment failure [8]. Therefore, identification and characterization of key molecules of chemoresistance are of utmost importance.

AKT is a family of three serine/threonine kinases, AKT1, AKT2, and AKT3, located on chromosomes 14, 19, and 1, respectively [9]. Among the three isoforms, AKT1 has been found to be associated with aggressive behavior and drug resistance in a variety of human cancers, such as pancreatic, prostate, ovarian, and thyroid cancers [10,11]. Moreover, previous studies have shown that AKT1 overexpression plays a critical role in colorectal cancer metastasis, and AKT1 downregulation reverses chemoresistance in p53-mutated colon cancer cells [12]. In light of these findings, we investigated the effect of AKT1 knockdown on the progression of HCC cells and on the sensitivity of HCC cells to the chemotherapy drug EPI [13]. EPI has been widely used throughout the world for liver cancer therapy in the past few years, as well as for therapy for other kinds of cancers, including lung cancer, bladder cancer, and breast cancer [14,15,16], by intercalating DNA strands into a complex formation that inhibits DNA and RNA synthesis.

AKT1 may play a crucial role in the drug resistance of liver cancer cells. The aim of this study is to investigate the effect of knocking down AKT on the proliferation and apoptosis of liver cancer cells, and to explore the effect of AKT1 on the chemosensitivity of EPI. This study is expected to explore the function of AKT1 in cancer and provide potential targets for the treatment of liver cancer.

2 Materials and Methods

2.1 Cell culture and transfection

The liver cancer cell lines HepG2, SMMC-7721, and

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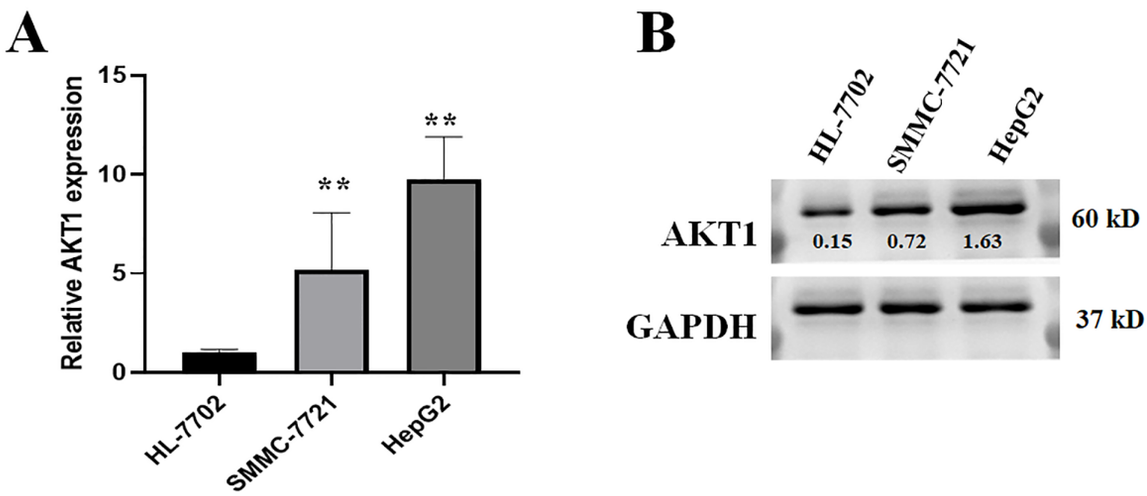

Figure 1. The expression of AKT1 in liver cancer cell lines.

(A) ATK1 was highly expressed in the mRNA of the normal liver cell line HL-7702 and the liver cancer cell lines SMMC-7721 and HepG2, as found using RT-qPCR, where ** means P < 0.01. (B) AKT1 was also highly expressed in the protein in the normal liver cell line HL-7702 and the liver cancer cell lines SMMC-7721 and HepG2, as found using Western blot analysis. The protein level was normalized to GAPDH.

normal cell line HL-7702 (Cell Bank, Chinese Academy of Sciences, Shanghai, China) were cultivated in an RPMI-1640 media supplement with 10% fetal bovine serum (FBS) in a 37°C incubator with 5% CO2. To silence and overexpress AKT1, the HepG2 and SMMC-7721 cells were transfected with small interfering RNA (siRNA) (Santa Cruz Biotechnology, Dallas, TX, USA; catalog number: sc-29195, their AKT1 is hereafter referred to as "AKT1-RNAi") and with the vector of pEGFP-N1-AKT1 (plasmid was synthesized by GenePharma, Shanghai, China; their AKT1 is hereafter referred to as "pEGFP-N1-AKT1"). A blank (Control-siRNA (catalog number: sc-37007) or pEGFP-N1) group was used as the control. For the transfection of the HepG2 and SMMC-7721 cells in the overexpression groups and the siRNA groups, the cells were seeded in 6-well plates. When the cells reached 70–80% confluency, the cells were transfected with the siRNA or plasmids (6 μg) using lipofectamine 2000, following the manufacturer's instructions. Primary antibodies AKT1 (#2938), Cyclin D1 (#2922, 1:1000), Bcl-2 (#3498, 1:1000), PTEN (#9559, 1:1000) and GAPDH (#5174, 1:1000) were purchased from cell signaling technology.

2.2 Quantitative real-time polymerase chain reaction (qRT-PCR)

Table 1 Primers information

Gene	Forward primer	Reverse primer	Products length
AKT1	5'-CATC-GCTTCTTTGCCGG-TAT-3' (Tm: 59°C)	5'-TTGGTCAGGTG-GTGTGATGGT-3' (Tm: 60°C)	152bp
PTEN	5'-GCGTGCAGATA-ATGACAAGGAA-3' (Tm: 59°C)	5'-GGATTTGACG-GCTCCTCTACTG-3' (Tm: 59°C)	150bp
GAPDH	5'-CATGTTCGTCAT-GGGTGTGAA-3' (Tm: 59°C)	5'-GGCATGGACT-GTGGTCATGAG-3' (Tm: 60°C)	154bp

The HepG2, SMMC-7721, and HL-7702 cells (2×105) were thoroughly lysed Trizol reaction and total RNA was extracted. Complementary DNA (cDNA) was prepared using the Reverse Transcription System (Takara, Dalian, China) and stored at -20°C. After dilution of the cDNA sample, qPCR was performed

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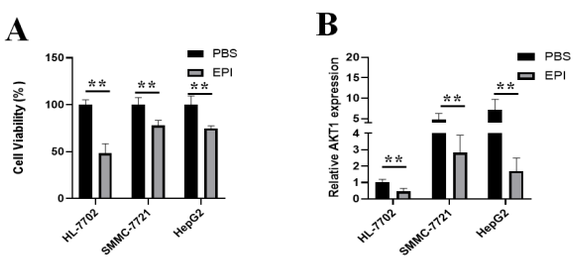

Figure 2. EPI downregulates the expression of AKT1 in liver cancer cells.

(A) Cell viability after EPI treatment in different cell lines. Cells were treated with EPI (3 μM) for 24 h, and the cell viability was detected using MTT assay. n = 3 and ** means P < 0.01. (B) Relative expression level of AKT1 after EPI treatment of cells in different cell lines, where n = 3 and ** means P < 0.01.

(PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were incubated in a blocking liquid (5% (w/v) non-fat milk) at 4°C for 60 min and washed with TBST for three times. Then, the membranes were incubated with primary antibodies at 4°C overnight. After three times of washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Protein bands were detected using an enhanced chemiluminescence detection kit (Sigma-Aldrich, St. Louis, MO, USA).

2.4 Cell proliferation assay

MTT assay was used to study the role of AKT1 in the proliferation of the HepG2 and SMMC-7721 cells. Briefly, 5×103 cells were seeded in a 96-well plate 24 h before transfection. The transfected cells (SMMC-7721 and HepG2) were incubated for 12 h, 24 h, 48 h and 72 h.

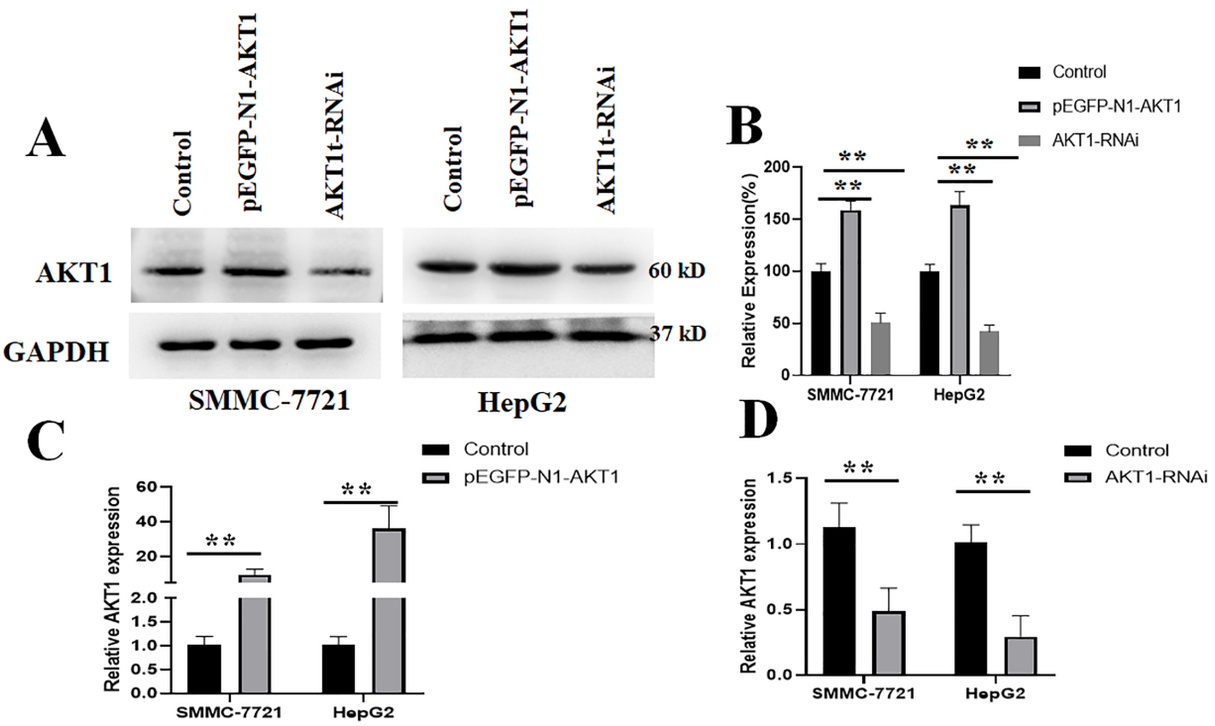

Figure 3. Potential downregulation or upregulation of AKT1 expression after cell transfection with RNAi and plasmid.

(A) Protein expression of ATK1 in the transfected cells. (B) Quantification of data in A, where n = 3 and * means P < 0.05. (C) Relative expression level of ATK1 after cell transfection with pEGFP-N1(Blank) or pEGFP-N1-AKT1, where n = 3 and * means P < 0.05. (D) Relative expression level of ATK1 after cell transfection with control-siRNA(Blank) or AKT1-RNAi, where n = 3 and ** means P < 0.01.

2.3 Western blot

Proteins were harvested and separated by SDS-PAGE and transferred onto a polyvinylidenedifluoride

After the treatment, 100 μl MTT (0.5 mg/mL, Beyotime, ST316) was added and incubated for 4 h, after r removing the supernatant and 150 μL DMSO was added to

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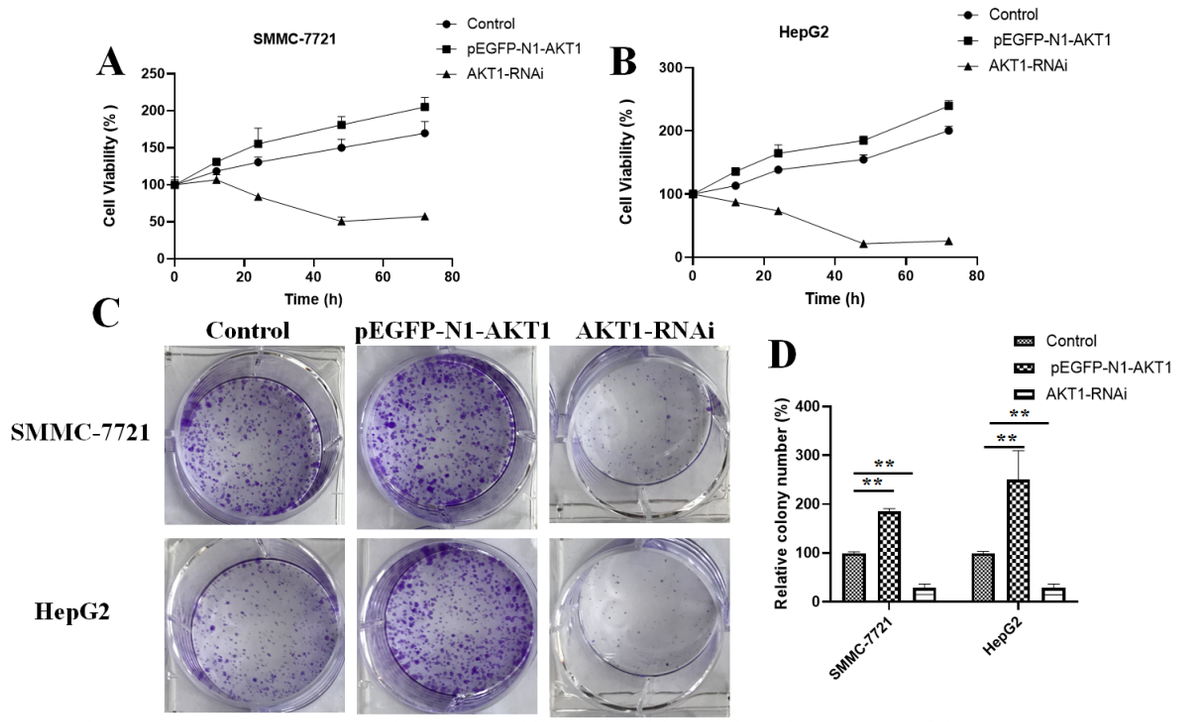

Figure 4. AKT1 knockdown inhibits the proliferation of SMMC-7721 and HepG2 cells.

(A) MTT absorbance of the SMMC-7721 cells with different treatments. (B) MTT absorbance of the HepG2 cells with different treatments. (C) Colony formation of SMMC-7721 and HepG2 cells with different treatments. (D) Relative cell colony numbers in C, where n = 3 and ** means P < 0.01.

each well. The plates were finally detected using a microplate reader at 490 nm. Experiments were performed in triplicate. The cell viability was calculated as follows:

Cell viability (%) = × 100%

2.5 Colony assay

The transfected cells (SMMC-7721 and HepG2) were seeded in 6-well plates (500cells/well) and incubated for 10 days to allow for colony formation. After staining with crystal violet, colonies containing more than 50 cells were counted and evaluated [17].

2.6 Cell cycle analysis

To detect the cell cycle distribution, the HepG2 and SMMC-7721 cells were transfected with pEGFP-N1-AKT1 or AKT1-RNAi. After 24 h, the cells were harvested and fixed in ice-cold 70 % methanol overnight. Then cells were stained with propidium iodide (PI; Sigma, USA) and analyzed using the FACScan flow cytometer (BD Biosciences, USA).

2.7 Annexin V-FITC/PI assay

The HepG2 and SMMC-7721 cells were transfected with pEGFP-N1-AKT1 or AKT1-RNAi for 24 h, cells were harvested and washed with PBS twice. After staining with Annexin V-FITC and PI (Beyotime, C1062M), cells were analyzed using the FACScan flow cytometer (BD Biosciences, USA).

2.8 Statistical analysis

All the data were analyzed using the SPSS 22.0 statistical software (IBM, Armonk, NY, USA). The results were expressed as means ± standard deviation (SD). The statistical analyses were done by the Student's t test, and comparisons of the means of 3 or more groups were performed using ANOVA with Tukey's post-hoc test using SPSS software. Differences with $P < 0.05$ were considered statistically significant.

3 Results

3.1 High AKT1 expression levels in the human liver cancer cell lines

qRT-PCR and western blotting were used to identify the expression level of AKT1 in human liver cancer cell lines. As shown in ure 1A, compared with normal human liver HL-7702 cells, the mRNA expression levels of AKT1 were increased about 10-times and 5-times in HepG2 cells and SMMC-7721 cells, respectively. The protein expression levels of AKT1 showed almost the same trends (Figure 1B). These results indicated that compared with HL-7702, the expression of AKT1

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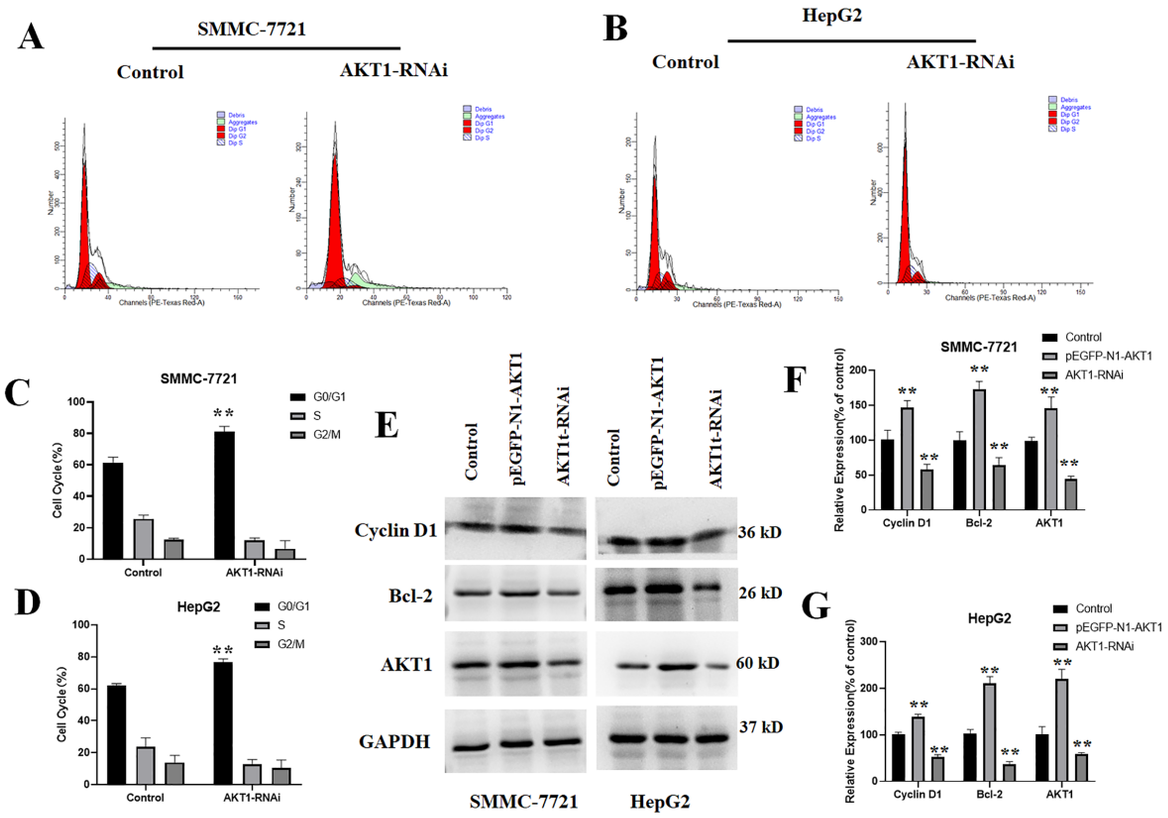

Figure 5. AKT1 knockdown blocks the cell cycle of SMMC-7721 and HepG2 cells.

(A) Cell cycle test of the SMMC-7721 cells after transfection with AKT1-RNAi. (B) Cell cycle test of the HepG2 cells after transfection with AKT1-RNAi. (C) Quantification of FACs data in A, where n = 3. (D) Quantification of FACs data in D, where n = 3 and ** means P < 0.01. (E) Western blot results for Cyclin D1, Bcl2, AKT1 and GAPDH in the SMMC-7721 and HepG2 cells. (F and G) Quantification of the data of Western Blots normalized to GAPDH, where n = 3 and ** means P < 0.01.

in cancer cell lines is significantly increased (P<0.01), indicating that AKT1 may be a necessary gene for cancer induction or cure.

3.2 EPI downregulates the expression of AKT1 in liver cancer cells

EPI has been widely used in liver cancer therapy as a chemotherapy drug but has often induced drug resistance. As shown in Figure 2A, after EPI treatment, only 50% of the HL-7702 cells were survived, while 75% of the SMMC-7721 and HepG2 cells were survived. Moreover, Figure 2B showed that EPI reduced the AKT1 expressions in all the treated cells, and the AKT1 expression level changes were negatively related to EPI-induced cell death. These results suggested that AKT1 mighty be one of the important factors of EPI-induced cell death.

3.3 Regulation of AKT1 expression in liver cancer cell lines through plasmid or RNAi transfection

pEGFP-N1-AKT1 and AKT1-RNAi transfection were used to overexpress and interfere with AKT1 expression in liver cancer cells, respectively. As shown in Figure 3C, after pEGFP-N1-AKT1 transfection, the mRNA expression levels of AKT1 in the SMMC-7721 and HepG2 cells were increased about 20-fold and 40-fold, respectively, ($p < 0.01$). A similar trend was detected in the protein in the SMMC-7721 and HepG2 cells using western blot analysis (Figure 3A and Figure 3B). Furthermore, the qRT-PCR and western blot results revealed that transfection with siRNA significantly suppressed the expression of AKT1 (Figure 3A, 3B, and 3D).

3.4 AKT1 knockdown inhibits the growth of SMMC-7721 and HepG2 cells

To investigate the function of AKT1 in the liver cancer cell line, we used the MTT assay and the colony formation assay to detect the proliferation of SMMC-7721 and HepG2 cells after the overexpress and interfere

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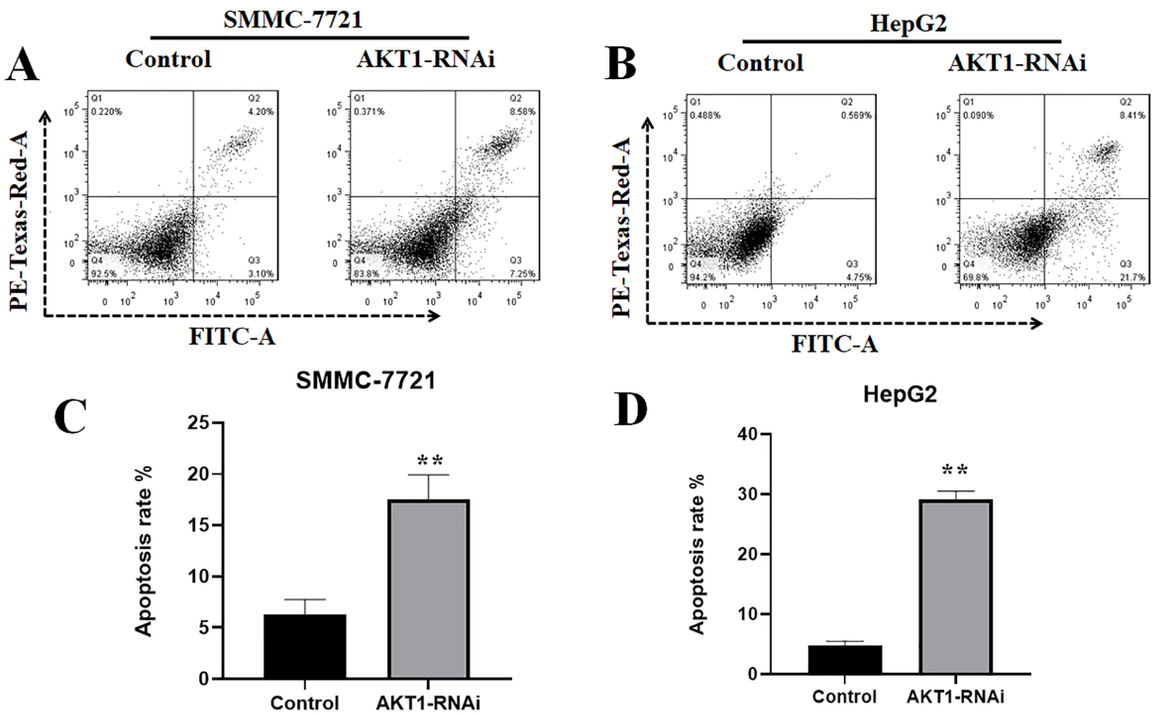

Figure 6. AKT1 knockdown induces apoptosis in SMMC-7721 and HepG2 cells.

(A) Cell apoptosis test of the SMMC-7721 cells after transfection with AKT1-RNAi. (B) Cell apoptosis test of the HepG2 cells after transfection with AKT1-RNAi. (C) Quantification of the FACs data in A, where n = 3 and ** means P < 0.01. (D) Quantification of the FACs data in B, where n = 3 and ** means P < 0.01.

of AKT1. As shown in Figure 4A, compared with the control group, AKT1 RNAi transfection significantly inhibited the proliferation of SMMC-7721 cells, while pEGFP-N1-AKT1 transfection showed induction of cell growth. Similar results were also observed in HepG2 cells (Figure 4B). These results suggested that knocking down AKT1 could suppress the proliferation of the SMMC-7721 and HepG2 cells. We further detected the cell colony numbers after transfection with AKT1-RNAi and pEGFP-N1-AKT1, as shown in Figure 4C and 4D, the number of colonies in SMMC-7721 and HepG2 cells overexpressed with AKT1 increased by nearly 2.2 times. While knocking down AKT1 resulted in a cell colony count of only 0.2 times that of the control group. The above results indicated that knocking down AKT1 can inhibit the proliferation of SMMC-7721 and HepG2 cells.

3.5 AKT1 knockdown blocks cell cycle of HepG2 and SMMC-7721 cells

To test how the AKT1 gene inhibits the proliferation of the liver cancer cells, we detected the effect of AKT1 knockdown on the cell cycle of HepG2 and SMMC-7721 cells. As shown in Figure 5A and 5C, after transfected with AKT1-RNAi, the proportion of cells in G0/G1 phase of SMMC-7721 cells were significantly increased to 78.72 % compared to 59.08 % in the control group. Also in HepG2 cells, AKT1 knockdown resulted in 78.13 % cell arrest in G0/G1 phase, higher than 61.42 % in the control group (Figure 5B and 5D).

We further measured the relative protein expression levels of CyclinD1 and Bcl-2, which are the well-known targets in the cell cycle, in both the HepG2 and SMMC-7721 cells. As shown in Figure 5E-G, in both HepG2 and SMMC-7721 cells, overexpression of AKT1 increased the expression of Cyclin D1 and Bcl-2. Meanwhile, knockdown the expression of AKT1 showed decreased expression of Cyclin D1 and Bcl-2. These results indicated that knockdown of AKT1 blocked liver cancer cells in G0/G1 phase.

3.6 AKT1 knockdown induces cell apoptosis in SMMC-7721 and HepG2 cells

The Annexin V-FITC/PI results showed that compared with the 7.32 % apoptosis ratio in the control group, the apoptosis ratio in the AKT1-RNAi transfection SMMC-7721 cells increased to 16.83 % (Figure 6A and 6C). In the HepG2 cells, AKT1-RNAi transfection increased the apoptosis ratio from 5.34 % to 29.84 % (Figure 6B and 6D). Interestingly, consistent with the results shown

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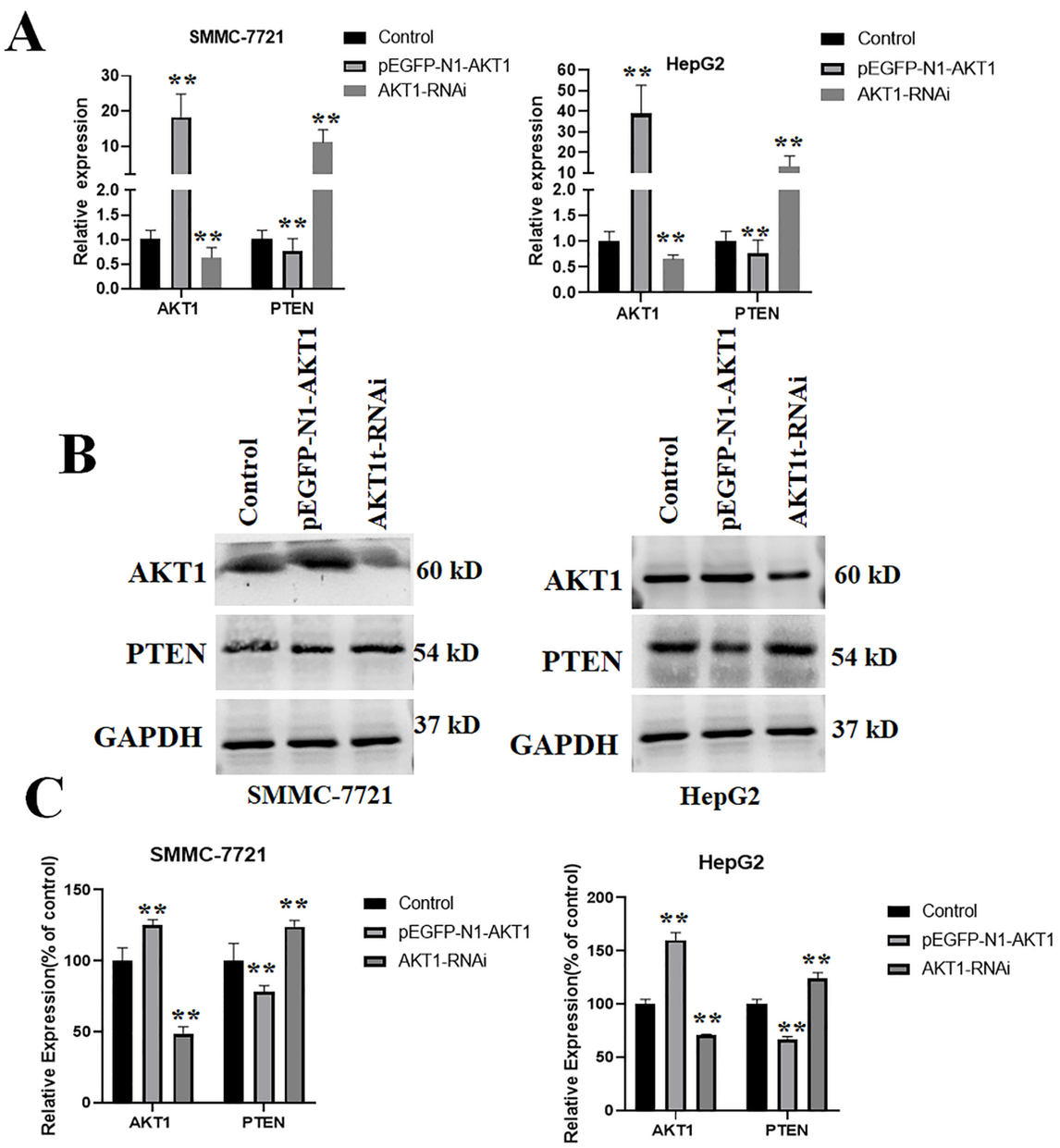

Figure 7. AKT1 knockdown increases PTEN expression in SMMC-7721 and HepG2 cells.

(A) mRNA relative expression level in the SMMC-7721 and HepG2 cells after transfection with pEGFP-N1-AKT1 and AKT1-RNAi, where n = 3. (B) Western blot data for PTEN, AKT1 and GAPDH in the SMMC-7721 and HepG2 cells. (C) Quantification of the data of Western Blots normalized to GAPDH, where n = 3 and ** means P < 0.01.

in Figure 1, the relative expression level of AKT1 in HepG2 cells was higher than that in SMMC-7721 cells. The number of cells that died after AKT1 interference was significantly increased, indicating that cancer cells with high AKT1 expression levels were more likely to die due to AKT1 intervention.

3.7 Negation correlation of the AKT1 expression level with the tumor suppressor gene PTEN in the liver cancer cells

To investigate the potential function of AKT1 in inhibiting liver cancer, we chose the tumor suppressor gene PTEN as the indicator, as it has been reported to have a lower expression level in liver cancer than in corresponding non-tumorous livers [18]. In SMMC-7721 cells, overexpression of AKT1 inhibited PTEN

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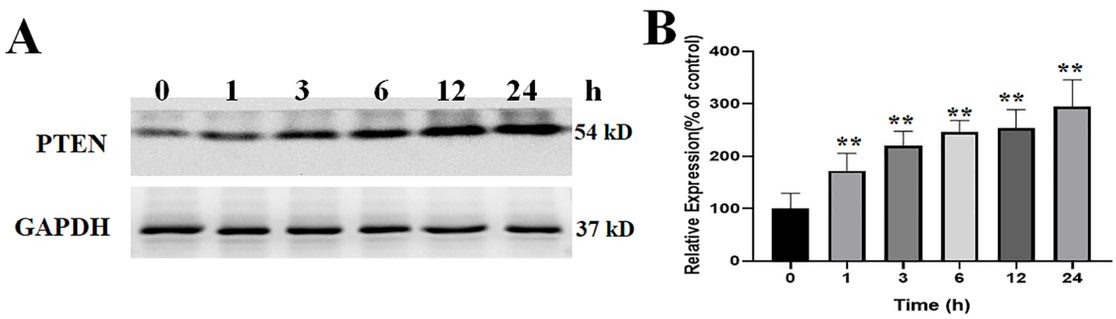

Figure 8. EPI stimulates the expression of PTEN in AKT1-RNAi HepG2 cells.
(A) Western blot analysis the expression of PTEN after treatment, where n = 3 and ** means P < 0.01. (B) Relative expression level of PTEN after EPI treatment, where n = 3 and ** means P < 0.01.

expression to 40 %, while ATK1 interference increased PTEN expression by approximately 17 times (Figure 7A). Furthermore, the western blot results showed that the PTEN protein level increased in AKT1 overexpression cells, while PTEN protein levels decreased to half of the previous expression levels in SMMC-7221 knockdown cells (Figure 7B and 7C). The negative expression relationship of AKT1 and PTEN was also found in both the mRNA and the protein in the HepG2 cells (Figure 7A–7C). These results indicated a negative correlation between AKT1 expression and PTEN expression, which suggested that controlling the expression level of AKT1 in tumor cells might be a key factor in tumor cell therapy.

3.8 EPI-induced time-dependent expression of PTEN in AKT1-RNAi HepG2 cells

Based on the findings on the effect of AKT1 knockdown on cell proliferation and apoptosis, we investigated whether HepG2 cells with suppressed AKT1 expression were more sensitive to EPI. Because the EPI suppressed the AKT1 expression, we further detected the PTEN protein expression level in the EPI-treated AKT1-RNAi HepG2 cells. Cell samples were harvested 0, 1, 3, 6, 12, and 24 h after treatment with 3 μM of EPI. The results showed that EPI stimulated the expression of PTEN and that the PTEN protein expression level increased about 2-fold only 3 h after the EPI treatment and about 3-fold after 24 h of EPI treatment (Figure 8). These results indicated that EPI increased the PTEN expression in cell lines with low AKT1 expression and that reducing the AKT1 expression may be a promising therapeutic method for enhancing the efficiency of EPI.

4.Discussions

Previous studies have shown that the occurrence and development of liver cancer is a complex process that involves multiple factors and genes. The abnormal regulation of multiple signaling pathways mediates the malignant biological behavior of liver cancer. It has now been clarified that the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway regulates cell growth, proliferation, survival, apoptosis, invasion, and migration, which plays an important role in the regulation of the cycle of tumor cells. The abnormal expression of the pathway is closely related to the occurrence and development of a variety of tumors [19,20,21,22]. It may also be related to EPI resistance, which often occurs during chemistry therapy in liver cancer. AKT can activate or inhibit its downstream target proteins such as Bad, Caspase9, and NF-κB through phosphorylation of the Forkhead (Fox) transcription factor family, mTOR, Par-4, P21, and others. However, cell proliferation induced by multiple growth factors can promote cell survival in a variety of ways [23,24].

Studies have found that AKT1 is necessary for the regulation of cell proliferation, differentiation, and apoptosis. Its activation is correlated with tumor occurrence, invasion, and metastasis [25,26]. In this study, we detected AKT1 expression in the human normal liver cell line HL-7702 and the liver cancer cell lines HepG2 and SMMC-7721. The results showed that the AKT1 expressions were increased in the HepG2 and SMMC-7721 cells than in the normal liver cell line, which may mean that the AKT1 gene is closely related to the proliferation and survival of liver cancer. Furthermore, we found that EPI efficiently caused the death of the cells with low AKT1 expression than of the cells with high AKT1 expression. Moreover, EPI reduced the AKT1 expression level in the liver cancer cells HepG2 and SMMC-7721. Therefore, this study has laid the foundation for further study of the effect of the AKT1 gene on the proliferation and apoptosis of liver cancer cells and on EPI resistance.

To further investigate the molecular biological mechanism of the AKT1 in liver cancer cells, we used pEGFP-N1-AKT1 plasmid and AKT1-RNAi to upregulate and downregulate AKT1 expression, respectively. The expression of AKT1 in the liver cancer

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cells transfected with pEGFP-N1-AKT1 plasmid was increased, whereas in liver cancer cells transfected with AKT1-RNAi, the AKT1 expression was decreased. We also observed that the expression of PTEN in the liver cancer cells transfected with AKT1-RNAi significantly increased. This indicated that PTEN might be the downstream genes regulated by AKT1 in HepG2 and SMMC-7721 cells.

AKT is a major kinase involved in cell survival and plays a role in the balance between cell proliferation and apoptosis. Knockdown of AKT1 is reported to be defective in growth and plays an important role in regulating tumor growth [27,28]. The main purpose of this study was to explore the possible molecular biological mechanism of AKT1 on the liver cancer cell lines HepG2 and SMMC-7721, especially on the proliferation, apoptosis, and cell cycle regulation of liver cancer cells. First, we examined the mRNA and protein expression of AKT1 in liver cancer cell lines. Compared with normal liver cell lines, AKT1 was highly expressed in HepG2 and SMMC-7721 cells. The expression of AKT1 in the HepG2 cells was the highest, followed by SMMC-7721, which were consistent with the results of previous studies in which AKT was highly expressed in cancer patients [29,30]. Our results showed that AKT1 silencing could induce the cell cycle arrest and apoptosis in HepG2 and SMMC-7721 cells, and that on the contrary, overexpression of AKT1 increase the proliferation of liver cancer cells. Thus, AKT1 may promote the proliferation and growth of liver cancer cells by inhibiting the apoptosis pathway and activation of the cell cycle.

Akt has been shown to be over activated in cancer cells through multiple mechanisms, including loss of PTEN, activation of PI3K [31]. On the other hand, studies have found that the hyperproliferation of PTEN-deficient cells is the cause of increased AKT phosphorylation [32]. Recent studies have shown that PTEN is a negative regulator of Notch1, which inhibits the growth of liver cancer cells by inhibiting the expression of MRP-1 [33,34]. In this study, we also found that the expression of the AKT1 in HepG2 and SMMC-7721 cells was negatively correlated with the expression of PTEN. These results confirmed our hypothesis that AKT1 negatively regulates PTEN expression and that the liver cancer cell lines HepG2 and SMMC-7721 can downregulate AKT1 gene expression by transfecting the cells with AKT1-RNAi to inhibit their survival and proliferation and by promoting their apoptosis. Previous studies have indicated that the PTEN gene is closely associated with tumor suppressor genes, which suggests the potential role of AKT1 in tumor cell therapy.

To further explore the mechanisms of AKT1-induced apoptosis and the relationship with the PI3K/AKT1/PTEN signaling pathway, we examined the effects of EPI on AKT1 and PTEN protein expression. The results showed that EPI inhibited the AKT1 gene and protein expression. Previous studies have shown that AKT1 plays a very important role in cell growth, differentiation, proliferation, and tumorigenesis [35], and it may have the same effects on tumor cells. However, after AKT1 was silenced, EPI increased the expression of the PTEN protein. As a tumor suppressor gene, PTEN increased its expression and inhibited the activity of PI3K/AKT, thereby increasing the inhibitory effect of EPI chemosensitivity [36].

5. Conclusions

In summary, our study found that EPI can induce a decrease in the expression of AKT1 mRNA and protein in liver cancer cells. Silencing AKT1 can enhance EPI induced cell apoptosis and G0/G1 cycle arrest, increase the expression of tumor suppressor gene PTEN, and induce liver cell death. This study suggests that in clinical practice, AKT1 inhibitors can be combined with chemotherapy drugs such as doxorubicin to increase the killing rate of liver cancer cells, reduce the incidence of chemotherapy resistance, and improve disease control rates.

Conflicts of interest

All the authors declare that they have no actual or potential competing financial interests.

Author contributions

H Si and W Xiao designed the project and wrote this paper. X Liu, L Wang, N Zhang and L Zhao performed the experiments. Y Jin and H Si analyzed the data.

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