AKT Phosphorylation Promotes Chemoresistance in Hepatocellular Carcinoma

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Research

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Abstract

Aim: This study was aimed to explore the effects of AKT phosphorylation in the chemoresistance and cell viability of hepatocellular carcinoma (HCC).

Materials & methods: We developed two taxol-resisted hepatocellualr cancer cells: TAX Resis HepG2 and TAX Resis SMMC7721.

Results: Phosphorylation of AKT on Thr 308 was highly expressed in taxol-resisted HepG2 and SMMC7721 cells. AKT phosphorylation manipulated by MK2206 and SC79 were correlated with cell viability. Downregulation of AKT phosphorylation promoted the apoptos is and suppressed the migration ability of taxol-resisted HepG2 and SMMC7721 cells, which also could be rescued by activate the AKT phosphorylation via SC79.

Conclusion: AKT phosphorylation manipulated by MK2206 and SC79 were correlated with cell viability, migration and apoptosis in taxol-resisted HCC cells.

Introduction

Hepatocellular carcinoma (HCC) is the most common subtype of liver cancer and the fourth most frequent cause of death around the world [1]. In the last few decades, the treatment of HCC has been gradually improved, but its mortality rate remains high [2]. Only less than 30% of the HCC patients are suitable for radical resection or liver transplantation, and systemic chemotherapy is required for advanced HCC patients [3]. However, chemoresistance often develops [4]. The possible mechanisms of chemoresistance include disorder of the critical signal pathways, changes of the targets of anticancer drugs, and increased drug efflux [5].

The activation of AKT kinase signaling pathway of human cancers is either indirectly through the activation of intersecting oncogenic pathways or directly through PI3 kinase, somatic mutation of PTEN, or AKT itself [6, 7]. The AKT kinase, in turn, activates numerous downstream targets which could boost tumor survival, growth, and progression. As a result, most human tumors are believed to rely on varying degrees of AKT signaling to maintain their viability. Overexpression and activation of AKT are usually associated with resistance to radiotherapy or chemotherapy [8, 9]. PTEN overexpression and PI3K inhibitors in PTEN-null cells have demonstrated the reversal of drug resistance [8, 10]. The cytotoxicity of chemotherapeutic agents could be enhanced by dominant-negative mutants of AKT, which suggests that AKT plays a pivotal role in drug resistance [10, 11]. Accordingly, small-molecule inhibitors of AKT suitable for clinical use have huge potential in the treatment of cancer [12, 13]. More recently, accumulating evidences also have proved that AKT phosphorylation is highly expressed or overexpressed in chemoresistance tumor samples. However, the expression pattern and possible roles of AKT phosphorylation in HCC have not been investigated.

In this work, we conducted this study to investigate a role of AKT phosphorylation in the taxol-resisted hepatocellualr cancer cells: TAX Resis HepG2 and TAX Resis SMMC7721. We firstly found that AKT phosphorylation is overexpressed in taxol-resisted hepatocellualr cancer cells. We also observed that AKT phosphorylation regulates the growth, apoptosis and migration of taxol-resisted hepatocellualr cancer cells.

Materials And Methods

Cell lines

Human HCC cell line HepG2 and SMMC-7721 were obtained from the Cell Bank of the Institute of Biochemistry and Cell Biology (Shanghai, China) in 2009 and maintained in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, Missouri, USA). These cell lines contained 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah, USA) and were placed at 37°C in 5% CO_2. All cell lines were freshened every two months and used them within 20 passages. These cell lines were identified by the growth profile and morphology examinations, and all of them are mycoplasma-free cell lines.

Real-time quantitative PCR

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from the tissue samples and cells. We conducted real-time quantitative PCR (abbreviated as qPCR) in SYBR Green PCR Master Mix (Roche Diagnostic GmbH, Mannheim, Germany) with a total volume of 20 μL. We conducted all the reactions in duplicate. The $2^{-\Delta\Delta CT}$ method was applied for normalization of Caspase3 and BCL-2 mRNA levels to GAPDH mRNA levels. The PCR primers for AKT phosphorylation were synthesized from Invitrogen. The sequences for BCL-2, Caspase3 and GAPDH were as follows: hBcl2_F: 5'-CAGGAAACGGCCCGGAT-3', hBcl2_R 5'-CTGGGGCCTTTCATCCTCC-3', hCaspase3_F: 5'-CTGCGGCTGGTGGAAGAG-3', hCaspase3_R: 5'- ATATGGGGCCTGAACAGCTC-3', hGAPDH_F: 5'-GCACCGTCAAGGCTGAGAAC-3' and hGAPDH_R: 5'- TGGTGAAGACGCCAGTGGA-3'.

Western blot

Lysis buffer containing protease inhibitors (Promega, Madison, WI) was used for the extraction of total protein. We separated the proteins using 12% SDS-PAGE gel, then transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, USA). The membrane was blocked with 5% non-fat milk, then incubated with anti-AKT total antibody (1:1000, 9272, Cell signaling technology, USA), anti-AKT phosphorylation [P-AKT (T308)] antibody (1:1000, 13038, Cell signaling technology, USA), or anti-GAPDH (1:2000, Sigma-Aldrich, St. Louis, MO, USA). Then we washed the membrane extensively and incubated with secondary antibody (Pierce, IL, USA). ECL reagents were applied for the detection of the proteins.

Immunofluorescence

We placed round glass slides on the bottom of the wells of a 24-well plate and coated them with gelatin, then we seeded cells at a density of 1×10^4 cells per well. We incubated the slides initially with primary

antibody against AKT phosphorylation [P-AKT (T308)] antibody (1:1000, 13038, Cell signaling technology, USA) at 4℃ in a humidified chamber for a night. Afterthat we added goat antimouse secondary antibody (labeled with FITC, 1:500 dilutions; Life Technologies) and incubated them for 1 h. Finally, we observed the slides under a fluorescence microscope (Nikon) and captured the images using the NIS-Elements BR 4.20.00 software.

MTT assay

Taxol-resisted HepG2 and SMMC7721 hepatocellualr cancer cells were treated with MK-2206 (S1078, Selleck, USA) 9 μM for 72 hours (MK2206 group) and rescued with SC79 (HY18749, MCE, China) 1μg/Ml for 1 hour after removed MK2206 prior to analysis (MK2206 + rescued with SC79 group). The evaluation of cell proliferation was conducted using the MTT in accordance with manufacturer's instructions. In short, we added 10 μL of MTT solution to the culture medium, and incubated them for another 4 h. At 490 nm wavelength was the absorbance determined.

Cell apoptosis

Taxol-resisted HepG2 and SMMC7721 hepatocellualr cancer cells were processed with MK-2206 (9 μM) for 72 hours (MK2206 group) and rescued with SC79 (1μg/mL) for 1 hour after removed MK2206 prior to analysis (MK2206 + rescued with SC79 group). The measurements of apoptosis and necrosis in drug-treated cells were conducted using an Annexin-V– fluorescein isothiocyanate/propidium iodide (FITC/PI) apoptosis detection kit (KeyGEN Biotech, Nanjing, China) in accordance with the manufacturer's instruction, with the help of an FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). Viable cells were stained negative for both Annexin-V and PI (Annexin-V-/PI-), while apoptotic cells were positive only with Annexin-V (Annexin-V+/PI-), late-stage necrotic and apoptotic cells were double-stained with Annexin-V and PI (Annexin-V+/PI+).

Cell migration assay

Fadu cells were grown to 50–70% confluence. We added the cells into the upper chamber of the insert (BD Bioscience, 8-μm pore size) for the migration assays. We incubated the cells in medium without serum, yet with 10% FBS in the lower chamber for chemo attractant in both assays. After a few hours of incubation, we wiped out the cells which did not migrate through the pores carefully with cotton wool. Finally, we stained the cells with 0.2% crystal violet and 20% methanol, and counted the imaged inserts.

Statistical analysis

SPSS 21.0 software package (SPSS, Inc., Chicago, IL, USA) was applied for statistical calculations. The evaluation of the differences between two groups was conducted using the Student *t*-test. And one-way analysis of variance was applied to analyze the comparison of the means greater than or equal to three groups. Data were presented as the mean ± standard error of the mean (abbreviated as SEM). $P < 0.05$ was defined as statistically significant.

Results

Establishment of taxol-resisted HepG2 and SMMC7721 hepatocellualr cancer cells

We developed two taxol-resisted HCC cells: TAX Resis HepG2 and TAX Resis HepG2 SMMC7721. To test the taxol resistance ability of these cells, we cultured wide type and taxol-resisted HepG2 or SMMC7721 with indicated concentrations of Taxol (from 2 to 1280 nM). To determin the cell viability under taxol cultured environment, MTT assay were performed. As showed in Fig. 1A and B, the 50% inhibitory concentration (IC50) in HepG2 and SMMC7721 wide type cells were 77.30 ± 5.67 and 166.39 ± 5.32, while in HepG2 and SMMC7721 TAX Resis type ones were 405.46 ± 5.69 and 577.49 ± 504, respectively. The resistance index (RI) of Taxol-resisted cells of HepG2 and SMMC7721 were calculated based on the 50% inhibitory concentration (IC50), RI = 5.25 ± 0.07 for HepG2 and RI = 3.47 ± 0.03 for SMMC7721 (Fig. 1C). The results indicated that these two TAX Resis types of HepG2 and SMMC7721 were successfully established.

Phosphorylation of AKT on Thr 308 was highly expressed in taxol-resisted HepG2 and SMMC7721 cells.

To investigate whether phosphorylation of AKT was highly expressed in TAX Resis types of HepG2 and SMMC7721 cells, western blot analysis were applied to analyze the phosphorylation levels of AKT on Thr 308 [p-AKT(T308)] and AKT (in total) (Fig. 2A). We found that the average phosphorylation of AKT on Thr 308 [p-AKT (T308)] protein level in TAX Resis types of HepG2 and SMMC7721 cells was significantly higher than that in their wide types. Moreover, we performed immunofluorescence analysis of p-AKT (T308) in wide type and taxol-resisted HepG2 or SMMC7721 hepatocellualr cancer cells. The results showed that the expression of phosphorylation of AKT on Thr 308 [p-AKT (T308)] was significantly increased in TAX Resis types of HepG2 and SMMC7721 cells as compared with that in their wide types (Figure. 2B). Together, these results suggested that phosphorylation of AKT on Thr 308 were highly expressed in taxol-resisted HepG2 and SMMC7721 cells.

AKT phosphorylation manipulated by MK2206 and SC79 were correlate with cell viability in Taxol-resisted HepG2 and SMMC7721 cells

To investigate whether the expression level of AKT phosphorylation were correlated with cell viability, we manipulated the AKT phosphorylation level by the inhibitor (MK2206) and activator (SC79). Firstly, western blot analysis demonstrated that MK2206 decreased the level of AKT phosphorylation while SC79 activate the level of AKT phosphorylation in the dose response manner in HepG2 and SMMC7721 (Fig. 3A and 3B). To measure the function of AKT phosphorylation downregulation and up regulation on the viability and proliferation ability of the Taxol-resisted HepG2 and SMMC7721 cells, MTT assays were performed. Taxol-resisted HepG2 and SMMC7721 hepatocellualr cancer cells were processed with MK-2206 (9 μM) for 72 hours (MK2206 group) and rescued with SC79 (1μg/mL) for 1 hour after removed MK2206 prior to analysis (MK2206 + rescued with SC79 group). MTT assay showed that AKT phosphorylation downregulation group (MK2206 group) were significantly inhibited the viability of the Taxol-resisted HepG2 and SMMC7721 cells compared with mock group and downregulation was rescued

with SC79 group (Fig. 3C, *P< 0.05). These results indicated that the AKT phosphorylation manipulated by MK2206 and SC79 were correlate with cell viability in Taxol-resisted HepG2 and SMMC7721 cells.

Downregulation of AKT phosphorylation promoted the apoptosis of Taxol-resisted HepG2 and SMMC7721 cells

Since phosphorylation of AKT on Thr 308 was highly expressed in Taxol-resisted HepG2 and SMMC7721 cells, we would wonder whether high phosphorylation of AKT could reduce the apoptosis to resist the chemotherapy. To evaluated the apoptosis status, Taxol-resisted HepG2 and SMMC7721 hepatocellualr cancer cells were processed with MK-2206 (9 μM) for 72 hours (MK2206 group) and rescued with SC79 (1μg/mL) for 1 hour after removed MK2206 prior to analysis (MK2206 + rescued with SC79 group), and Annexin-V–PI assay were conducted. As showed in Fig. 4A, the upper right quadrant (Q2; Annexin-V+/PI+) implied apoptosis, AKT phosphorylation downregulation group (MK2206 group) were significantly promoted the apoptosis of the Taxol-resisted HepG2 and SMMC7721 cells compared with mock group and downregulation was rescued with SC79 group. The percentages of apoptosis parts were indicated in Fig. 4B. Furthermore, we have analyzed the expression level of two genes related with apoptosis (BCL-2 and Caspase3). BCL-2 were significantly reduced in AKT phosphorylation downregulation group (MK2206 group) compared with mock group and downregulation was rescued with SC79 group (Fig. 4C, **P< 0.01). While caspase3 were dramatically increased in AKT phosphorylation downregulation group (MK2206 group) compared with mock group and downregulation was rescued with SC79 group (Fig. 4D, **P< 0.01). Together, these results indicated that downregulation of AKT phosphorylation promoted the apoptosis of Taxol-resisted HepG2 and SMMC7721 cells, which also could be rescued by activate the AKT phosphorylation via SC79.

Regulation of AKT phosphorylation correlate with the migration potential of Taxol-resisted HepG2 and SMMC7721 cells

To further explore the influence of AKT phosphorylation on the migration potential of the Taxol-resisted HepG2 and SMMC7721 cells, transwell assays were used. The data showed that the number of migratory cells in the AKT phosphorylation downregulation group (MK2206 group) significantly reduced compared with mock group and downregulation was rescued with SC79 group (Fig. 5A and 5B, *P< 0.05). These data suggested that the downregulation of AKT phosphorylation suppressed the migration ability of Taxol-resisted HepG2 and SMMC7721 cells, which also could be rescued by activate the AKT phosphorylation via SC79.

Discussion

Although anticancer drugs like taxol, have been widely applied in the treatment of HCC, chemoresistance is still an important therapeutic difficulty and its molecular mechanisms are poorly known. Our study

demonstrated that PI3K/Akt pathway might be related to the chemoresistance in HCC. Our results demonstrated that MK-2206 and SC79 efficaciously regulates Akt phosphorylation as well as inhibit the growth of taxol-resisted HepG2 and SMMC7721 cells. This finding is in accordance with previous studies that MK-2206 could efficaciously inhibit the growth of different cancer cells, for instance, lung, colorectal, nasopharyngeal, and thyroid *in vivo* and *in vitro* [14–17].

The activation of PI3K/Akt pathway plays a vital role in the biology of cancers, for instance, tumorigenesis, tumor metastasis, and the resistance to traditional chemotherapeutic agents [18]. The addition of paclitaxel was shown to increase Akt activity, which was in accordance with lower level of cell death [19]. Induction of Akt by cisplatin, as well, was in charge of the chemotherapeutic resistance observed [20]. Nevertheless, the exact mechanism by which Akt activation leads to chemoresistance is unknown. In this study, we demonstrated that AKT phosphorylation regulates chemoresistance in taxol-resisted HepG2 and SMMC7721 cells.

Previous studies have demonstrated that the inhibition of PI3K/Akt could increase the induction of apoptosis in colon cancer cells incorporated with irinotecan and sensitize ovarian cancer cells to paclitaxel [21]. Molecule-targeted drugs that target other points of PI3K/Akt pathway, for instance LY294002, have been demonstrated to be able to restore the sensitivity of hepatocellular cancer cells to chemotherapy in vitro [22, 23]. In the study, we found that AKT phosphorylation could be manipulated by two small molecule reagents MK2206 and SC79, which would further correlate with cell viability, migration and apoptosis.

Conclusion

AKT phosphorylation manipulated by MK2206 and SC79 were correlate with cell viability, migration and apoptosis in Taxol-resisted HepG2 and SMMC7721 cells, which may promote chemoresistance of HCC, and act as a valuable target for the treatment of HCC. This study provides important information for the identification and characterization of a new molecular target and a marker for HCC therapy.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests

None.

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Authors' contributions

Yunfei Duan conceived and designed the study. Xiaodong Li and Jing Chen performed the experiments. Yu Yang and Zhen Qu performed the analyses. Xiaodong Li and Yunjie Lu wrote the manuscript text. All authors reviewed and proved the manuscript.

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Figures

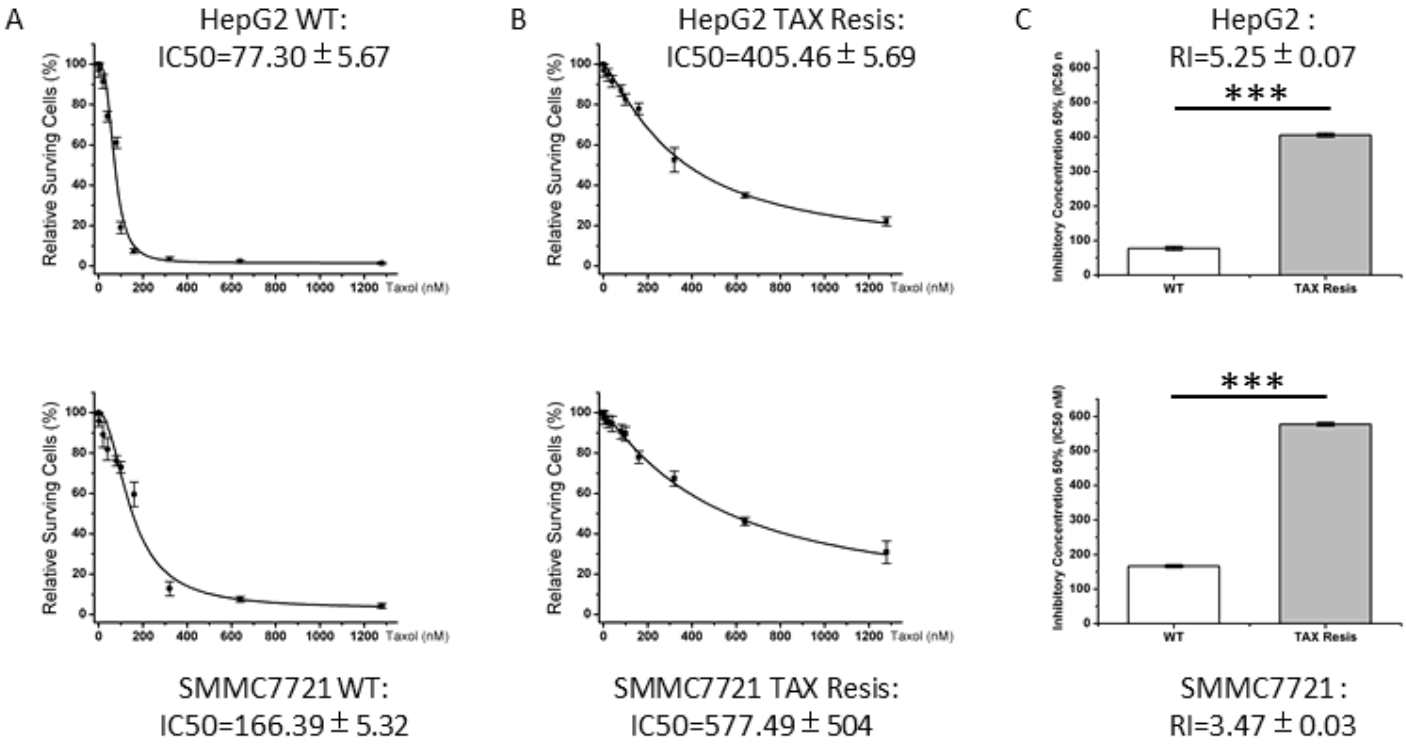

Figure 1

Influence of Taxol on the growth of wide type and taxol-resisted HepG2 and SMMC7721 hepatocellualr cancer cells. (A) Wide type cells or (B) taxol-resisted cells of HepG2 and SMMC7721 were treated 48h with specified concentrations of Taxol. Cell viability was measured using the MTT assay immediately. (C) Resistance index of taxol-resisted cells of HepG2 and SMMC7721 were calculated based on the the 50% inhibitory concentration (IC50). All experiments were performed three times. Significant differences are expressed by asterisk (*** P<0.001 versus wide type cells).

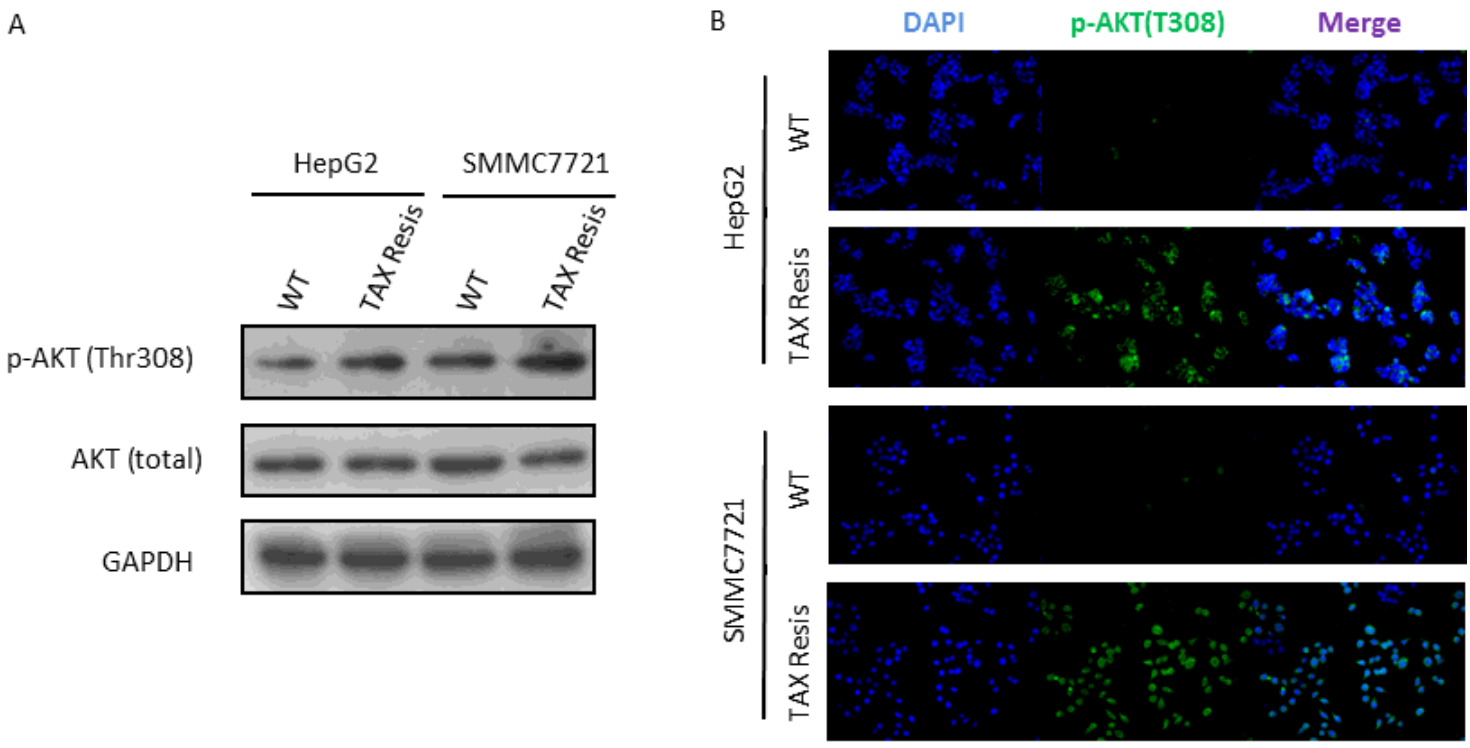

Figure 2

Expression level of phosphorylation of AKT on Thr 308 in taxol-resisted HepG2 and SMMC7721 hepatocellualr cancer cells. (A) Western blot analysis of Phosphorylation of AKT on Thr 308 (p-AKT(T308)) and AKT(total) in wide type and taxol-resisted HepG2 or SMMC7721 hepatocellualr cancer cells. GAPDH was set as a loading control. (B) Immunofluorescence analysis of p-AKT(T308) in wide type and taxol-resisted HepG2 or SMMC7721 hepatocellualr cancer cells. Blue: DAPI, Green: p-AKT(T308) detected by FITC-conjugated secondary antibody. Scale bars, 20 μm.

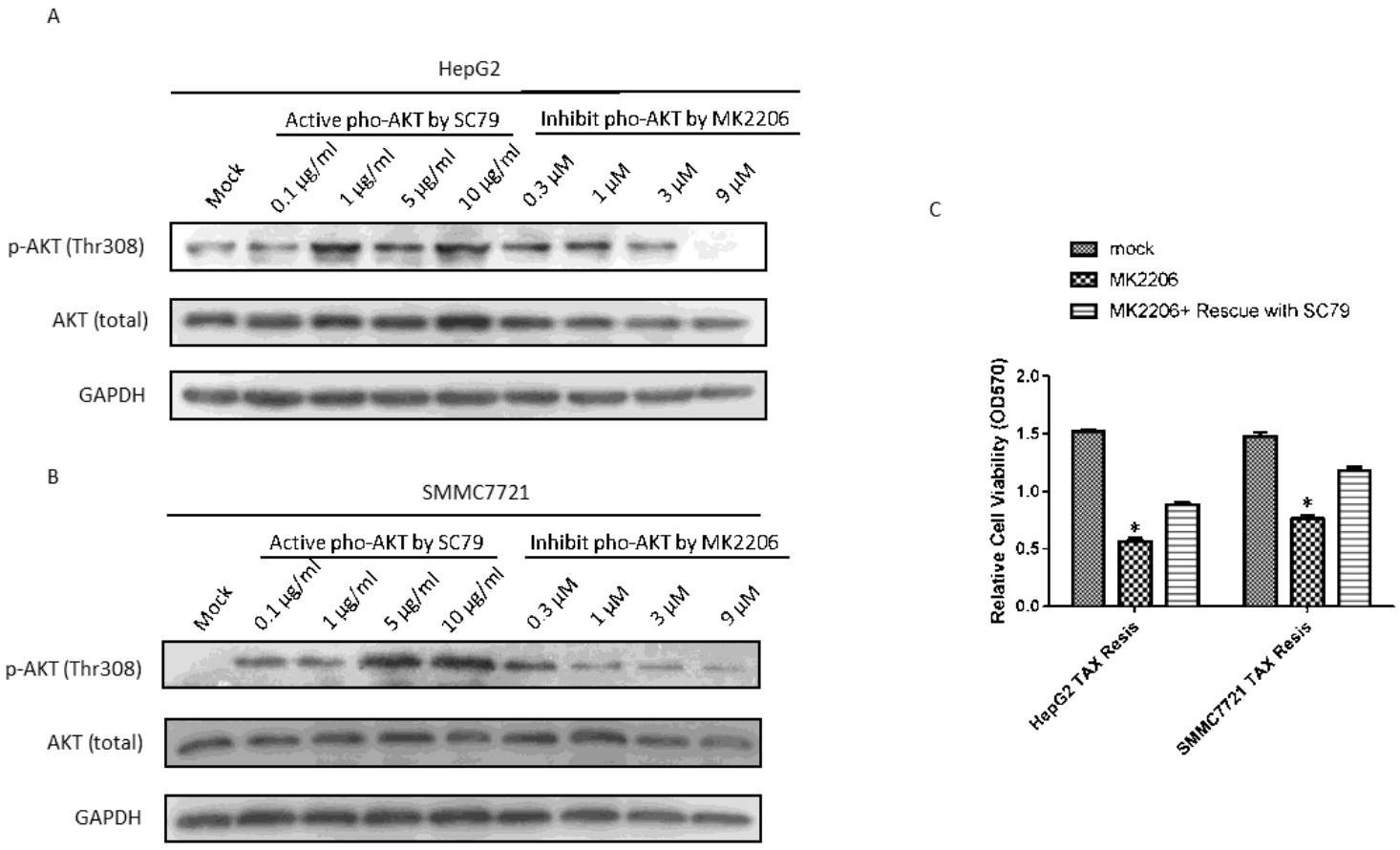

Figure 3

Effect of AKT phosphorylation manipulated by MK2206 and SC79 on cell viability in taxol-resisted HepG2 and SMMC7721 hepatocellualr cancer cells. (A) Western blot analysis of Phosphorylation of AKT on Thr 308 (p-AKT(T308)) and AKT(total) in the wide type of hepatocellualr cancer cells, which were dose responsed to the inhibitor (MK2206) and activator (SC79) of AKT phosphorylation. GAPDH was set as a loading control. (B) Cell viability was analyzed by MTT assay of inhibitor (MK2206) and activator (SC79) of AKT phosphorylation in taxol-resisted HepG2 and SMMC7721 hepatocellualr cancer cells. Taxol-resisted HepG2 and SMMC7721 hepatocellualr cancer cells were processed with MK-2206 (9 μM) for 72 hours (MK2206 group) and rescued with SC79 (1μg/mL) for 1 hour after removed MK2206 prior to analysis (MK2206 + rescued with SC79 group). All experiments were performed three times. Significant differences are expressed by asterisk (* $P<0.05$ versus mock and MK2206+SC79).

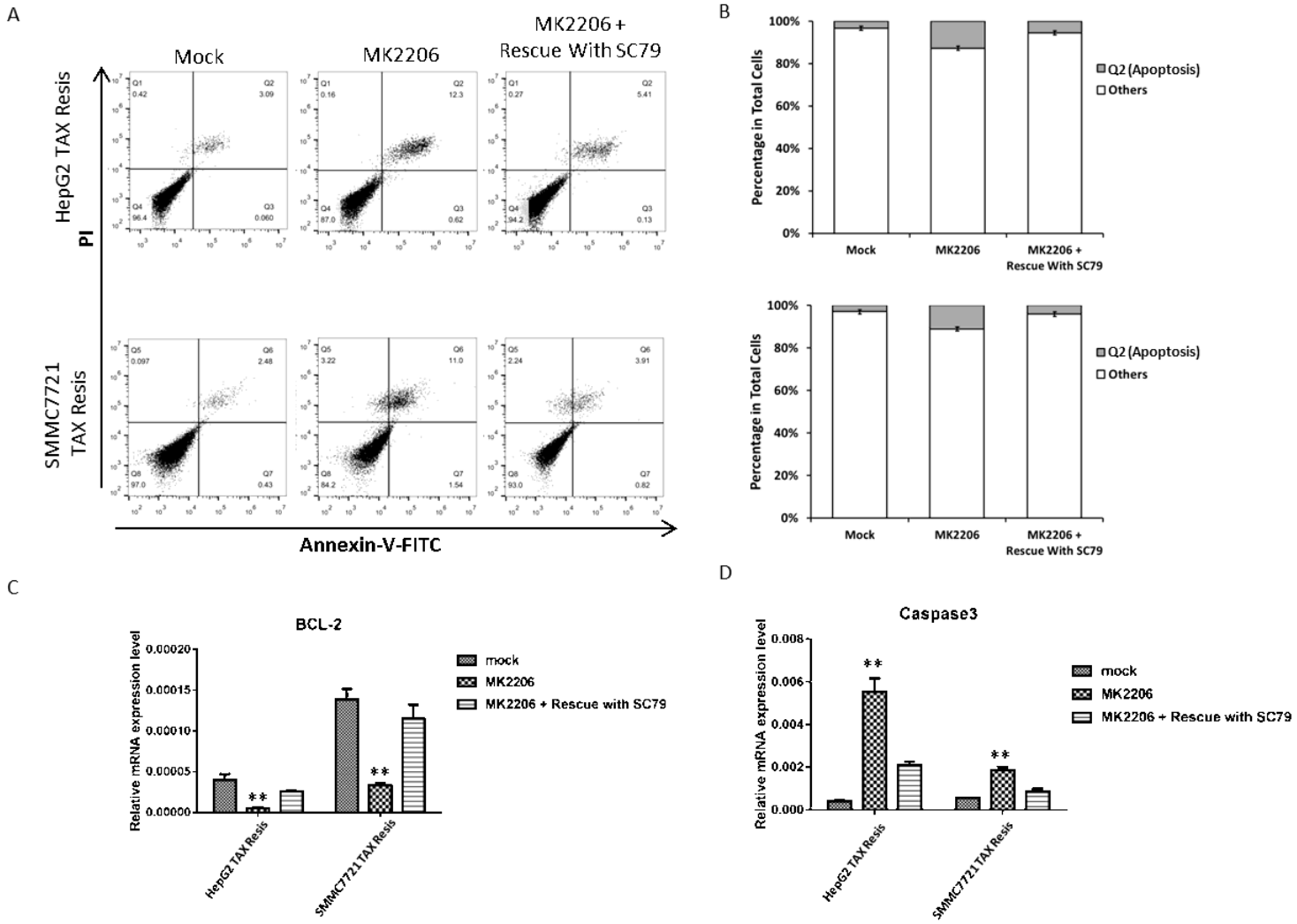

Figure 4

Effect of AKT phosphorylation manipulated by MK2206 and SC79 on apoptosis in taxol-resisted HepG2 and SMMC7721 hepatocellualr cancer cells. (A) Taxol-resisted HepG2 and SMMC7721 hepatocellualr cancer cells were processed with MK-2206 (9 μM) for 72 hours (MK2206 group) and rescued with SC79 (1μg/mL) for 1 hour after removed MK2206 prior to analysis (MK2206 + rescued with SC79 group). Annexin-V–PI assay was applied for the evaluation of apoptosis. The upper right quadrant (Q2; Annexin-V+/PI+) implied apoptosis. The experiment was conducted in triplicate and representative data were demonstrated. (B) Percentages were expressed in quadrants. Propidium iodide was abbreviated as PI. (C) Real-time quantitative PCR analysis of BCL-2 and Caspase 3 mRNA expression level at each treatment cells. The mRNA amounts were normalized to those of GAPDH mRNA. The means of three independent biological replicates are shown; error bars indicate the S.E.M. ** $P < 0.01$ by a one-tailed t-test.

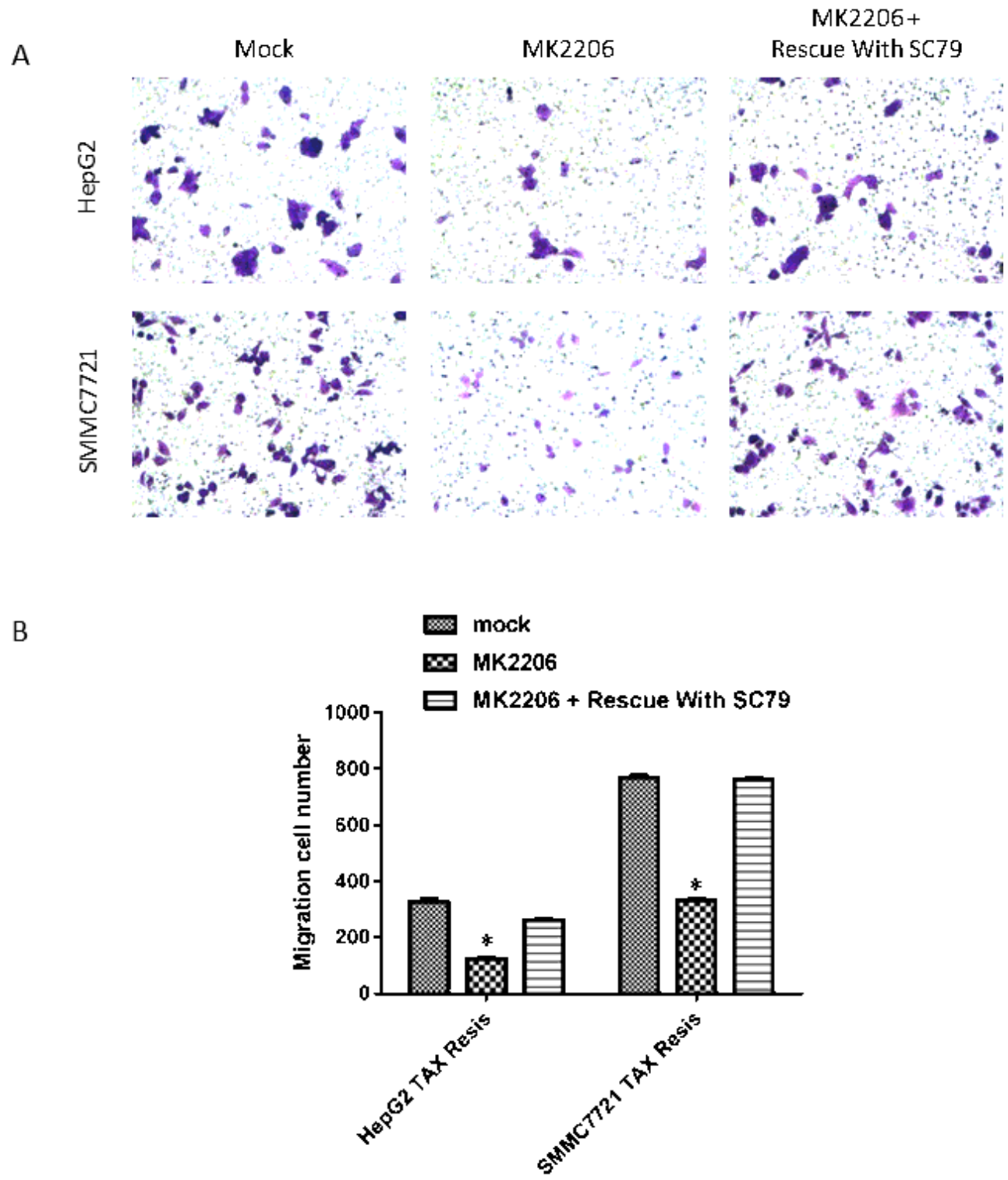

Figure 5

Effect of AKT phosphorylation manipulated by MK2206 and SC79 on migration in taxol-resisted HepG2 and SMMC7721 hepatocellualr cancer cells. (A) Typical images of the migration assays. Migration assay using 24-well Transwell system. Taxol-resisted HepG2 and SMMC7721 hepatocellualr cancer cells were processed with MK-2206 (9 μM) for 72 hours (MK2206 group) and rescued with SC79 (1μg/mL) for 1

hour after removed MK2206 prior to analysis (MK2206 + rescued with SC79 group). The migration cell numbers were counted on the lower surface; *$p < 0.05$.