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# Inhibition of PI3K/Akt/mTOR pathway by apigenin induces apoptosis and autophagy in hepatocellular carcinoma cells

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ABSTRACT

Apigenin is a dietary flavonoid with known antioxidant and antitumor effects against several types of cancers by promoting cell death and inducing cell cycle arrest. Apigenin also regulates a variety of intracellular signal transduction pathways during apoptosis or autophagy. However, the precise mechanism underlying the anticancer effects of apigenin in liver cancer remains poorly understood. In this study, we demonstrated that apigenin has anticancer activity against hepatocellular carcinoma cells. Apigenin inhibited the cell growth and induced cell death in a dose- and time-dependent manner in HepG2 cells. We found that apigenin treatment increased the expression of LC3-II and the number of GFP-LC3 puncta. Moreover, inhibition of autophagy with 3-MA and Atg5 gene silencing strengthened apigenin-induced proliferation inhibition and apoptosis. Our data has indicated that apigenin-induced autophagy has a protective effect against cell death. Additionally, apigenin induced apoptosis and autophagy through inhibition of PI3K/Akt/mTOR pathway. Most importantly, in vivo data showed that administration of apigenin decreased tumor growth and autophagy inhibition by 3-MA significantly enhanced the anticancer effect of apigenin. Collectively, our results reveal that apigenin inhibits cell proliferation and induces autophagy via suppressing the PI3K/Akt/mTOR pathway. Our results also suggest combination of autophagy inhibitors and apigenin would be a potential chemotherapeutic strategy against hepatocellular carcinoma.

## 1. Introduction

Hepatocellular carcinoma is the sixth most common cancer and ranked as the third leading cause of cancer-related deaths worldwide [1,2]. Due to lacking specific clinical symptoms and signs, early diagnoses and treatments, hepatocellular carcinoma become almost impossible [3]. The treatment of liver cancer includes surgical resection, local ablation, transplantation, transarterial embolization and immunotherapy [4]. Although improved diagnosis and therapeutic methods, the overall 5-year survival rate of approximately 12% [5]. Therefore, novel agents and effective therapeutic strategies to treat liver cancer are urgently required.

Apigenin (4′,5,7-trihydroxyflavone), is a natural flavonoid that is widely distributed in fruits and vegetables, such as parsley, orange, tea, chamomile and seasonings [6]. In recent years, apigenin has been shown to possess significant anti-inflammatory, anti-oxidant and anti-tumor properties [7]. Previous studies have reported that apigenin could inhibit the growth and proliferation, promote apoptotic cell death, induce cell cycle arrest and mitochondrial membrane potential disruption of cancer cells in vitro and in vivo [8–20]. However, the precise mechanism underlying the anti-tumor effects of apigenin remains poorly understood.

Autophagy, an evolutionarily conserved pathway, plays a vital role in degradation of the superfluous proteins and organelles. Autophagy is induced by a series of conditions, such as hypoxia, cellular stress and nutrient starvation [21]. Recent studies indicate that targeted autophagy may be an effective therapeutic strategy to fight cancer [22]. However, autophagy has dual roles in regulating cell survival with regard to cancer treatment [23]. Accumulating evidence suggests that in some conditions, cancer cells tend to use autophagy as a survival mechanism to avoid chemotherapeutic agents or γ-irradiation induced apoptosis [24]. Several molecular and signaling pathways play a crucial role in regulating autophagy in many types of cancers, such as the autophagy-related gene family [25] and PI3K/Akt/mTOR signaling pathway [26,27]. The mammalian target of rapamycin (mTOR) plays a critical role in regulating the balance between cell proliferation and autophagy in response to cellular stress induced by chemotherapeutics in cancer cells [28]. Previous studies have reported that apigenin could induce autophagy and apoptosis in breast cancer cells [29]. However, the relationship between apoptosis and autophagy induced by apigenin

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remains unknown.

In the present study, we aimed to investigate the effect of apigenin on apoptosis and autophagy in hepatocellular carcinoma cells and xenograft mouse model. Our results showed that apigenin induced apoptosis and autophagy in vitro and in vivo. We found that inhibition of autophagy led to inhibition of cell proliferation and tumor growth. Moreover, we also demonstrated that the apigenin induced autophagy and apoptosis via inhibiting PI3K/Akt/mTOR pathway.

## 2. Materials and methods

### 2.1. Reagents and antibodies

Apigenin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies for cleaved-caspase-3(1:1500), cleaved-caspase-9(1:1000), cleaved-PARP(1:1500), Bcl-2(1:1000), Bax(1:1000), PI3K(1:1500), p-PI3K(1:1000), Akt(1:1000), p-Akt(1:800), mTOR(1:1000), p-mTOR (1:800), Atg5(1:1000), Beclin1(1:1000) and SQSTM1(1:1500) were obtained from Cell Signal Technology (Boston, USA). Antibodies for LC3B(1:1000) and actin(1:5000) were purchased from Sigma (St. Louis, MO, USA). Atg5 siRNA, GFP-LC3B plasmid, insulin-like growth factor-1 and Ki-67 antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Annexin V-FITC and *propidium iodide* (PI) kit were obtained from Beyotime Company (Beijing, China). 3-MA, Bafilomycin A1, LY294002 and Rapamycin were purchased from Sigma (St. Louis, MO, USA).

### 2.2. Cell culture and treatment

The hepatocellular carcinoma cell line, HepG2, was purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). The HepG2 cells were cultured in DMEM medium (HyClone, Logan, Utah, USA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, Utah, USA) and 1% (v/v) penicillin/ streptomycin. Cells were grown in a humidified atmosphere containing 5% CO2 at 37 °C. At 80% confluence, the HepG2 cells were treated with Apigenin at different concentrations (0, 10, 20, 40 μM) for 12 h or with 20 μM Apigenin for various periods (0, 6, 12, 24 h).

### 2.3. Cell viability and death assay

Cell viability was measured using Cell Counting Kit-8 (Dojindo Molecular Technologies, Shanghai, China) according to the manufacturer's instructions. Briefly, $1 \times 10^4$ cells were seeded into 96-well plates. After treatment, 90 μl of medium and 10 μl of CCK-8 solution were added to each well and incubated at 37 °C for 2 h. Absorbance at 450 nm was measured using a Microplate Reader. The results are expressed as a percentage of the control. The cell death was performed using the trypan blue exclusion assay (Beyotime, Beijing, China). HepG2 cells were seeded in the 6-well plates and treated with Apigenin at different concentrations. After treatment, cells were harvested and stained with 0.4% trypan blue for 3 min. Viable cells were counted using an automated cell counter. Cell death ratio (%) is calculated as percentage of the dead cell number/the total cell number.

### 2.4. Detection of apoptosis

Apoptosis was determined by the Annexin V-FITC and propidiumiodide (PI) kit (Beyotime, Beijing, China) following the manufacturer's instructions. After treatment, cells were harvested and incubated with Annexin V-FITC and PI at room temperature in the dark for 15 min. Subsequently, the cell suspensions were immediately evaluated by flow cytometry.

### 2.5. Caspase-3 activity assay

The activity of the caspase-3 enzyme was assessed using the caspase-3 activity assay kit (Beyotime, Beijing, China) according to the manufacturer's protocol. After treatment, the cells were collected and lysed with lysis buffer. Cell lysate were incubated with assay buffer and caspase-3 substrate at 37 °C for 2 h. Absorbance at 405 nm was determined using a Microplate reader. The caspase-3 activity was normalized by the protein content of each sample and presented as a percentage of the control.

### 2.6. Plasmid and siRNA transfection

After seeding the cells in 6-well plates and incubation for 24 h, cells were transiently transfected with GFP-LC3 plasmid or Atg5-targeting small siRNA (Santa Cruz,Shanghai, China) using Lipofectamine 2000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions. After 24 h, cells were washed and treated with or without 20 μM Apigenin for 12 h and collected for further experiments. To assess autophagy, the cells that overexpressed GFP-LC3 were observed with a Zeiss confocal laser scanning microscope (Carl Zeiss, Shanghai, China).

### 2.7. Real-time PCR

Total RNA was extracted from the HepG2 cells using Trizol reagent (TaKaRa) and reverse-transcribed using a cDNA reverse transcription according to the manufacturer's instructions. The Atg5 primers used in this study were (5′-GGGTCCCTCTTGGGGTACAT-3′) and (5′- ACCACA CATCTCGAAGCACA-3′); the GAPDH primers were (5′-TGACAACAGC CTCAAGAT-3′) and (5′-GAGTCCTTCCACGATACC-3′). The relative expression levels of Atg5 were normalized to the mRNA of the GAPDH.

### 2.8. Tumorigenicity in nude mice

Five-week-old male BALB/c nude mice were purchased from the Shanghai Laboratory Animal Center of Chinese Academy Sciences and housed under specific pathogen-free condition. All animal experimental procedures were approved by the Animal Care and Use Committee of the Nanchang University, Nation Fortune Word No. 545 [2001]. HepG2 cells ($5 \times 10^6$) were re-suspended in 200 μl PBS and were injected subcutaneously into the lower right side flanks of mice to establish tumors. After 6 days of initial implantation, mice were randomly divided into control, 3-MA, apigenin and combination groups (n = 5 per group). Mice were intraperitoneally injected with apigenin (100 mg/kg) or 3-MA (1 mg/kg) every 3 days. The dose and administration approach were selected according to previous studies [30]. Tumor volume were measured with calipers every 3 days and tumors volumes (V) were calculated using the formula: V = 1/2 length × (width)2. All mice were sacrificed at 27 days and the tumors were dissected and weighed.

### 2.9. Immunohistochemistry analysis

Fixed tumor tissues were embedded in paraffin and were sectioned at a thickness of 4 μm. After rehydration and antigen retrieval, tumor sections were incubated with Ki-67 (1:100 dilution) at 4 °C overnight. Sections were washed and subsequently incubated with biotin-conjugated secondary anti-mouse IgG for 30 min at room temperature and visualized using diaminobenzidine according to the manufacturer's instructions.

### 2.10. Western blot analysis

HepG2 cells were homogenized in protein lysate buffer and c entrifuged at 12,000 *g* for 15 min at 4 °C. Protein concentrations were quantified by using the Bradford protein assay kit (Beyotime, Beijin, China). The protein samples were separated by SDS-PAGE, transferred

to polyvinylidene fluoride membranes and blocked with fresh 5% nonfat milk for 2 h at room temperature. The membranes were incubated with specific primary antibody overnight at 4 °C. After washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized by using Super Signal West Pico blotting kit (Pierce, Massachusetts, USA).

### 2.11. Statistical analysis

All data were presented as mean ± SEM from three independent experiments. Student's t test or one-way ANOVA analysis was used to compare the different treatment groups by using GraphPad Prism 5. $P < 0.05$ was considered to indicate a statistically significant difference.

## 3. Results

### 3.1. Apigenin inhibited cell growth and induced apoptosis in hepatocellular carcinoma cells

To investigate the effect of apigenin on the viability of hepatocellular carcinoma cells, we treated HepG2 cells with apigenin We found that apigenin inhibited cell viability in a time- and dose-dependent manner (Fig. 1**A** and **B**). Moreover, Annexin V and PI staining assay showed that apigenin induced cell death in a dose-dependent manner (Fig. 1**C** and **D**). To further explore the mechanism by apigenin induced cell death in HepG2 cells, we detected the anti-apoptotic and pro-apoptotic protein expression. We found that apigenin significantly increased the cleaved-caspase-3, cleaved- caspase-9, cleaved-PARP and Bax expression, while decreasing the expression of Bcl-2 (Fig. 1**E**). Similarly, apigenin significantly increased the caspase-3 activity in HepG2 cells (Fig. 1**F**).

### 3.2. Apigenin triggered autophagy in hepatocellular carcinoma cells

To investigate whether autophagy is involved in the cytotoxicity of apigenin, we first examined the processing of full-length LC3-I to LC3-II, an autophagy marker, in apigenin-treated HepG2 cells. We found that apigenin treatment increased the protein levels of LC3-II in a dose-dependent manner (Fig. 2**A**). However, we couldn't ascertain whether the increase of LC3B-II levels was due to activation of autophagy or blockade of autophagy-lysosomes fusion. Thus we next detected protein levels of SQSTM1, a selective substrate of autophagy, for activation of the autophagic flux lead to lysosomes degradation. Immunoblot analysis showed that apigenin treatment decreased the level of SQSTM1 (Fig. 2**A**), confirming that apigenin enhances the autophagic degradation process. Moreover, we found that apigenin treatment also significantly increased the expression of autophagy-related proteins, Atg5 and Beclin-1in HepG2 cells (Fig. 2**A**). As autophagy is a dynamic process, we used autophagy inhibitor (3-MA) to further confirm apigenin-induced autophagy in HepG2 cells. Our results showed that 3-MA suppressed the apigenin induced LC3-II accumulation (Fig. 2**B**) and decreased GFP-LC3-positive autophagosomes in the cells (Fig. 2**D**). Furthermore, Bafilomycin A1 (Baf A1), a specific inhibitor of the vacuolar-type H + -ATPase, significantly enhanced apigenin induced LC3-II accumulation (Fig. 2**C**). We also observed that Baf A1 resulted in increased the percentage of cells expressing GFP-LC3 positive autophagosomes in HepG2 cells that were treated with apigenin (Fig. 2**D**). Taken together, our findings demonstrated that apigenin treatment triggered autophagic flux in HepG2 cells.

### 3.3. Inhibition of autophagy enhance apoptosis induced by apigenin in hepatocellular carcinoma cells

Since autophagy has dual roles in regulating cell survival and cell death, we next investigated the effect of apigenin-induced autophagy on apoptosis in HepG2 cells. We found that pretreatment with 3-MA markedly attenuated apigenin induced LC3-II accumulation, increased the cleaved-caspase-3, cleaved-caspase-9, cleaved-PARP and Bax expression, while decreasing the Bcl-2 expression (Fig. 3**A**). Moreover, the CCK-8 assay result showed that the inhibited cell growth effects of apigenin were enhanced when treatment with apigenin was combined with 3-MA (Fig. 3**B**). Similarly, inhibition of autophagy significantly enhanced apigenin induced caspase-3 activity and cell death in HepG2 cells (Fig. 3**C** and **D**). In addition, combined treatment with 3-MA and apigenin significantly increased the population of annexin V-positive cells (Fig. 3**E**) compared with apigenin treatment alone. To further confirm autophagy was involved in apigenin-induced apoptosis, we suppressed autophagy using siRNA against Atg5. Atg5 targeted siRNA significantly downregulated Atg5 mRNA and protein expression in HepG2 cells (Fig. 3**F–H**). As expected, the increasing in LC3-II accumulation was significantly attenuated by Atg5 siRNA following exposure to 20 μM apigenin for 12 h (Fig. 3**I**). In particular, Atg5 siRNA treatment efficiently enhanced the apigenin induced cleaved-caspase-3, cleaved-caspase-9, cleaved-PARP and Bax expressions (Fig. 3**I**). Moreover, the apigenin-induced decrease in cell viability was significantly strengthened after treatment with Atg5 siRNA (Fig. 3**J**). Furthermore, knockdown of Atg5 resulted in remarkably increased caspase-3 activity and cell apoptosis induced by apigenin (Fig. 3**K–M**). Taken together, these results suggest that apigenin-induced autophagy has a protective role against apoptosis and suppression of autophagy enhance apoptosis in HepG2 cells.

### 3.4. Apigenin-induced apoptosis and autophagy through inhibition of PI3K/Akt/mTOR pathway

The PI3K/Akt/mTOR signaling pathway plays an important role in regulating cell cycle, proliferation, apoptosis and autophagy. To investigate the role of PI3K/Akt/mTOR pathway in apigenin-induced apoptosis and autophagy, the activation of PI3K, Akt and mTOR was examined by Western blot assay using phosphorylated antibodies. As shown in (Fig. 4**A**), treatment with apigenin decreased the levels of phosphorylated PI3K, Akt and mTOR in HepG2 cells. To confirm that the apigenin induced apoptosis and autophagy via the PI3K/Akt signaling pathway, we exposed the cells to PI3K inhibitor (LY294002) before treating with apigenin. Our results demonstrated that pretreated with LY294002 significantly enhanced apigenin induced p-Akt reducing (Fig. 4**B**). Moreover, pretreated with LY294002 significantly strengthened LC3II accumulation compared to only apigenin treated cells (Fig. 4**B**). The CCK-8 assay results showed that the inhibited cell growth effects of apigenin were enhanced when treatment with apigenin was combined with LY294002 (Fig. 4**C**). In addition, inhibition of PI3K significantly increased the number of apoptotic cells induced by apigenin (Fig. 4**D**). To further assess the effects of PI3K/AKT pathway in the anticancer activity of apigenin. Cells were pretreated with PI3K activator insulin-like growth factor-1 (IGF-1, 10 μM) for 2 h and incubated with apigenin (20 μM) for another 12 h. Our results demonstrated that PI3K activator significantly attenuated the inhibitory effects of apigenin on PI3K, Akt and mTOR phosphorylation (Fig. 4**E**). Moreover, the anti-proliferative and pro-apoptotic effects of apigenin were reversed by the PI3K activator (Fig. 4**F** and **G**). To ascertain whether mTOR is involved in apigenin-induced apoptosis and autophagy, mTOR expression was down-regulated in HepG2 cells by pretreatment with rapamycin. Combined treatment with rapamycin and apigenin significantly increased LC3II accumulation (Fig. 4**H**), inhibited cell viability (Fig. 4**I**) and increased the population of the number of apoptotic cells (Fig. 4**J**) compared with apigenin treatment alone. These data suggest that apigenin induces autophagy and apoptosis by inhibiting PI3K/Akt/mTOR pathway.

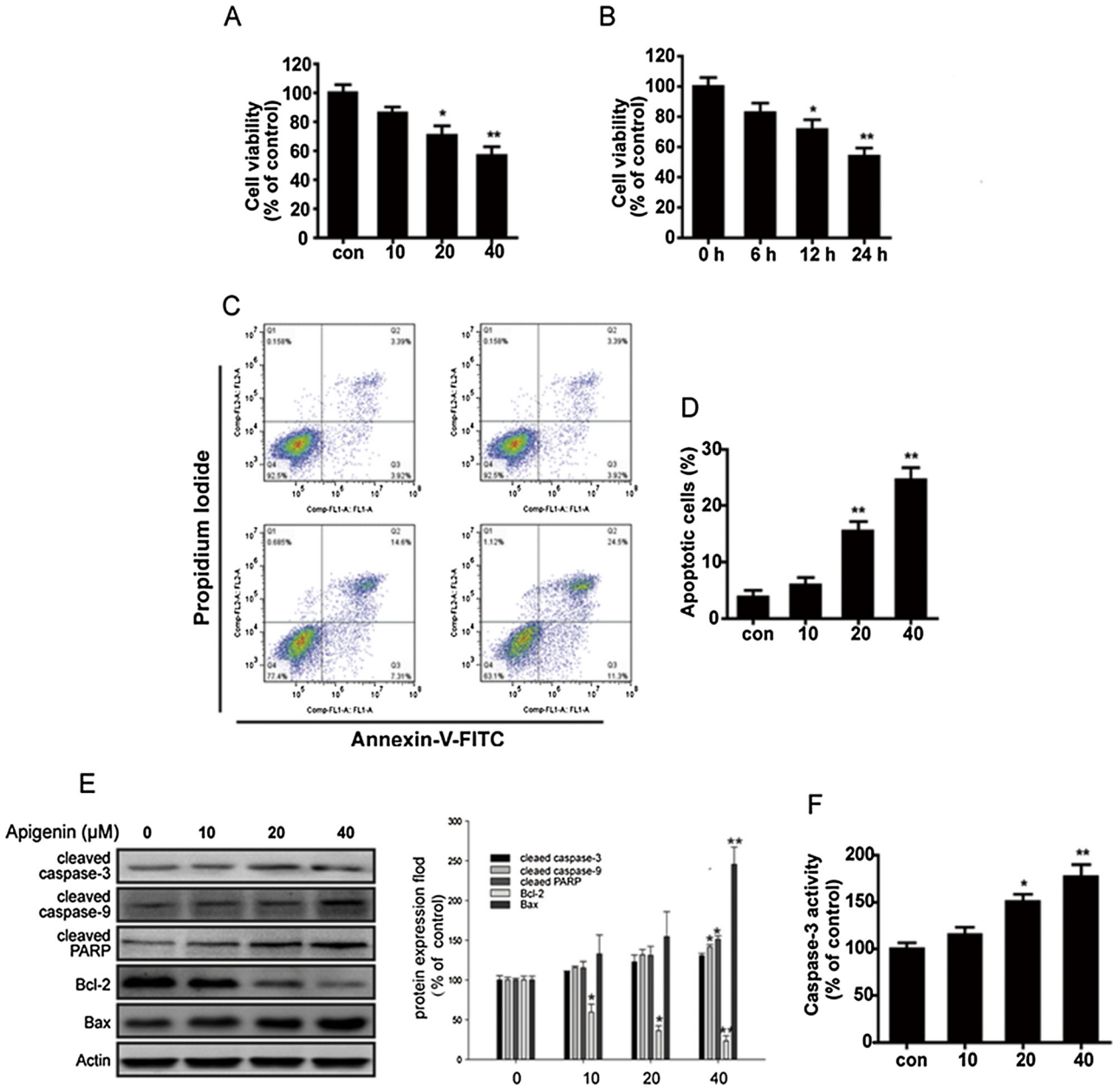


**Fig. 1. Apigenin inhibited cell growth and induced apoptosis in HepG2 cells.** (A) Cells were incubated with various concentrations (0, 10, 20 and 40 μM) of apigenin for 12 h. Cell viability was determined by using CCK-8 assay. (B) Cells were treated with 20 μM apigenin for 0, 6, 12, and 24 h. Cell viability was measured via CCK-8 assay. (C, D) Cell death were examined by annexin V/PI double stained assay. The results are expressed as a percentage of the control, which is set at 100%. (E) Cells were incubated with indicated apigenin concentration for 12 h. Apoptosis-related proteins expression levels were determined by Western blot analysis. (F) Caspase-3 activity was determined as described in material and methods. The values are presented as the means ± SEM at least three independent experiments, * $p < 0.05$, ** $p < 0.01$ versus the control group.

### *3.5. Apigenin inhibited the tumor growth in vivo*

To determine whether apigenin inhibits tumor growth in vivo, the xenograft model in male BALB/c nude mice was established. As shown in Fig. 5**A**, apigenin administration inhibited tumor growth and decreased tumor volume (Fig. 5**B**) and tumor weight (Fig. 5**C**) compared with the control group. There was no significant difference of body weight in all groups (Fig. 5**D**). Interestingly, co-treatment of 3-MA (autophagy inhibitor) with apigenin resulted in significantly smaller tumors, decreased tumor volume and tumor weight. Immunoblotting results revealed that apigenin treatment significantly increased LC3-II protein expression in tumors compared to the control group, whereas 3-MA treatment markedly attenuated apigenin induced LC3-II protein expression (Fig. 5**E**). In addition, the expression level of the pro-apoptotic protein Bax was significantly upregulated in the apigenin treatment group and 3-MA significantly enhanced apigenin-induced Bax protein expression (Fig. 5**E**). Furthermore, a significant decrease in cellular proliferation determined by Ki-67 immunohistochemistry was observed in the tumors treated with apigenin, and 3-MA treatment significantly strengthened apigenin-induced inhibition of cell proliferation (Fig. 5**F**). These results suggested that autophagy inhibition may sensitize tumors to apigenin through reducing tumor cell proliferation.

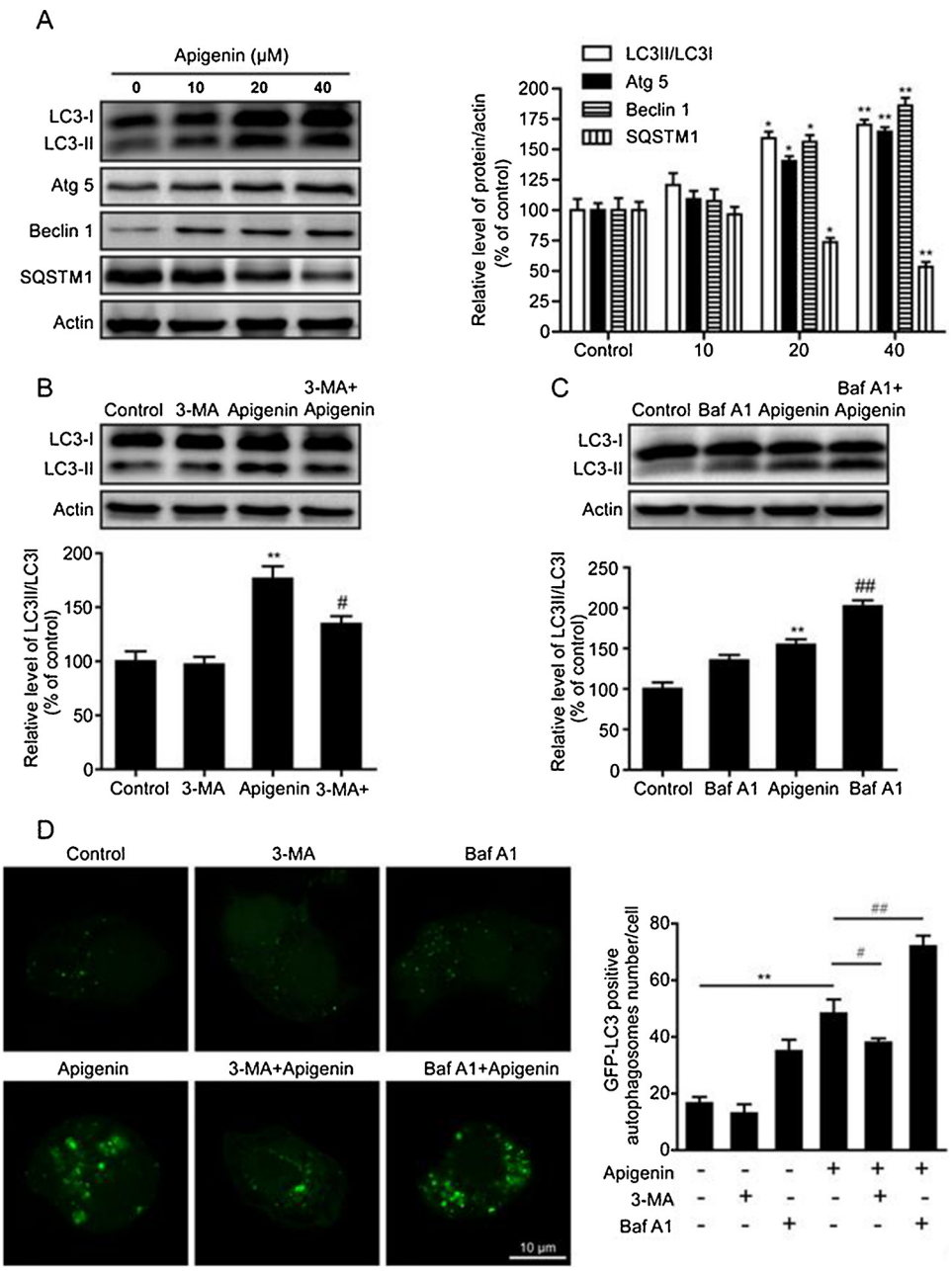


**Fig. 2. Apigenin induced autophagic flux in HepG2 cells.** (A) Cells were incubated with various concentrations (0, 10, 20 and 40 μM) of apigenin for 12 h. LC3, Atg5, Beclin1 and SQSTM1 expression levels were analyzed by western blot. (B) Cells were pretreated with 10 mM 3-MA and incubated with 20 μM apigenin for another 12 h. Expression of LC3 was analyzed by western blot. (C) Cells were incubated with apigenin (20 μM) in the absence or presence of Baf A1 (10 nM) for 12 h. Expression of LC3 was analyzed by western blot. (D) The GFP-LC3 puncta was examined using confocal microscopy and was quantified. The results are expressed as a percentage of the control, which is set at 100%. The values are presented as the means ± SEM at least three independent experiments. * $p < 0.05$, ** $p < 0.01$ versus the control group, # $p < 0.05$, ## $p < 0.01$ versus the apigenin (20 μM) group.

## 4. Discussion

Apigenin, one of the common natural flavonoids, has been demonstrated to inhibit tumour cell proliferation, angiogenesis and induce apoptosis in various types of tumor cells [31]. Our previous study has been demonstrated that apigenin inhibits hepatoma cell growth both in vitro and in vivo, which is associated with induction of cell cycle arrest and apoptosis [32]. However, the special mechanism underlying the anti-tumor effects of apigenin in hepatoma cell is still not well understood. In the present study, we found that apigenin inhibited cells growth and induced apoptosis in HepG2 cells. We also showed that apigenin treatment increased LC3-II expression, autophagolysosome vacuoles formation and triggered autophagic flux. Moreover, inhibition of autophagy led to inhibit proliferation and enhance apoptosis induced by apigenin in HepG2 cells. Additionally, we determined that apigenin causes autophagy and apoptosis through suppressing of PI3K/Akt/mTOR pathway. Most importantly, in vivo data showed that administration of apigenin decreased tumor growth and inhibition autophagy with 3-MA significantly enhanced the anticancer effect of apigenin.

Autophagy is an evolutionarily conserved process for engulfing damaged proteins and organelles, clearing subcellular debris and maintaining cellular homeostasis [33]. Induction of autophagy by antitumor reagents and chemotherapeutics has been well studied in a series of cancer models in vitro and in vivo [34,35]. In this study, we found that apigenin triggered autophagy, including induce autophagosomes formation, LC3 redistribution and autophagy related proteins expression. Recent evidences indicate that autophagy may act as a “double-edged sword” and has dual roles in regulating cell survival and proliferation [36]. Accumulating evidences have shown that autophagic cell death (type II programmed cell death), plays a crucial role in the anti-tumor activity of drugs and chemicals [37,38]. In cancer cells, autophagy is acted as a survival mechanism to resist chemotherapeutic agents induced apoptosis [39]. Previous studies have been shown that the suppression of autophagy leads to apoptosis, thus enhancing antitumor activity [40,41]. In our study, we observed that apigenin induced apoptosis and autophagy in a dose-dependent manner. Inhibition of

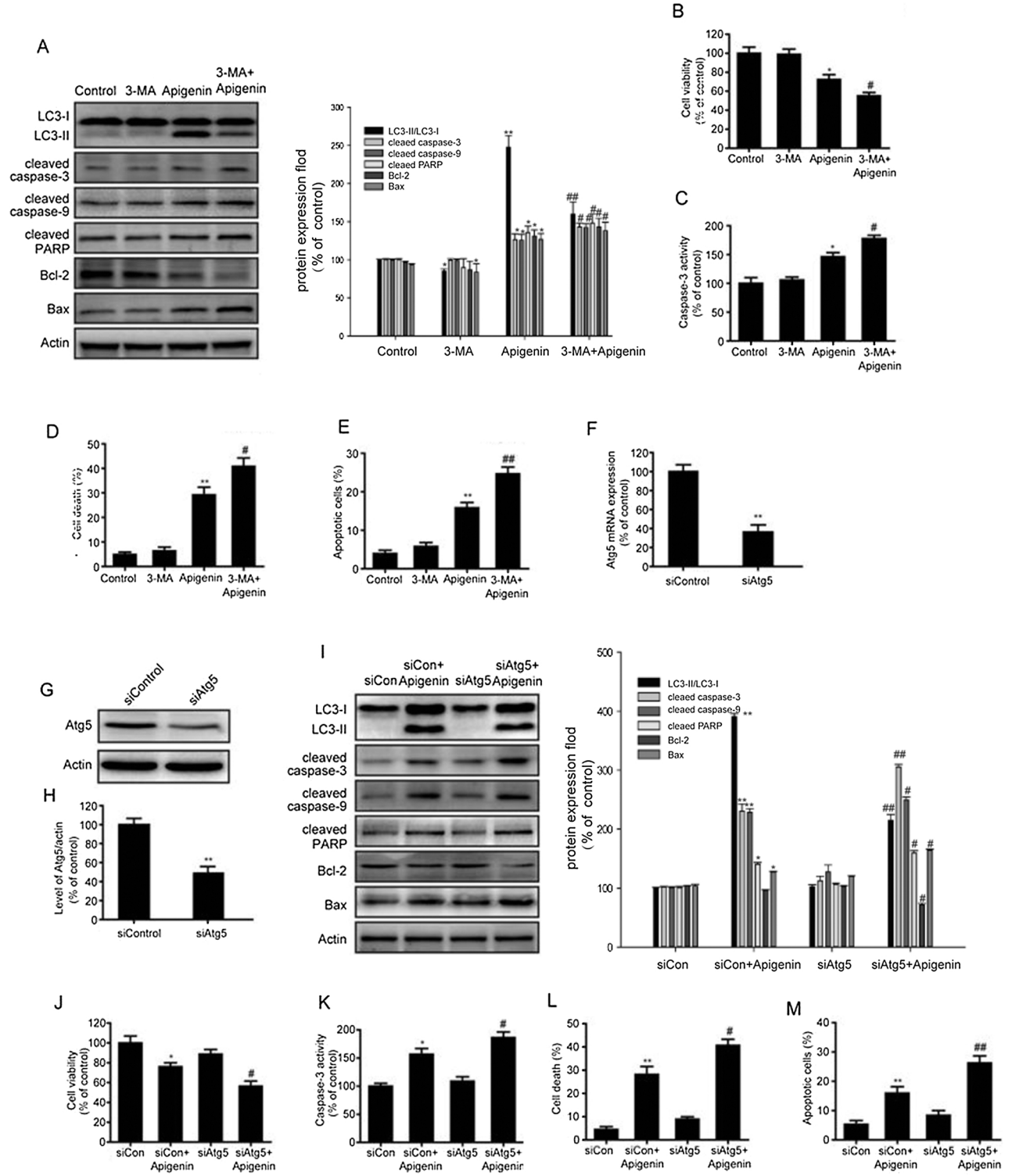


**Fig. 3. Autophagy decreased apigenin-induced apoptosis in HepG2 cells.** Cells were pretreated with 3-MA (10 mM) for 2 h and then incubated with apigenin (20 μM) for 12 h. (A) Cell lysates were subjected to western blotting. (B) Cell viability was determined by using CCK-8 assay. (C) Caspase-3 activity was determined as described in material and methods. (D) The cell death was performed using trypan blue exclusion assay. (E) Cell apoptosis were examined by flow cytometry. (F) Atg5 mRNA and protein (G and H) expression were examined after transiently transfected with Atg5-targeting small siRNA using Lipofectamine 2000. (I) Cells treated with the combination of apigenin (20 μM) and siAtg5 duplexes. Cell lysates were subjected to western blotting. (J) Cell viability was determined by using CCK-8 assay. (K) Caspase-3 activity. (L) The cell death was performed using trypan blue exclusion assay. (M) Cell apoptosis were examined by flow cytometry. Data are presented as the mean ± SEM of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$ versus the control group, # $p < 0.05$, ## $p < 0.01$ versus the apigenin group.

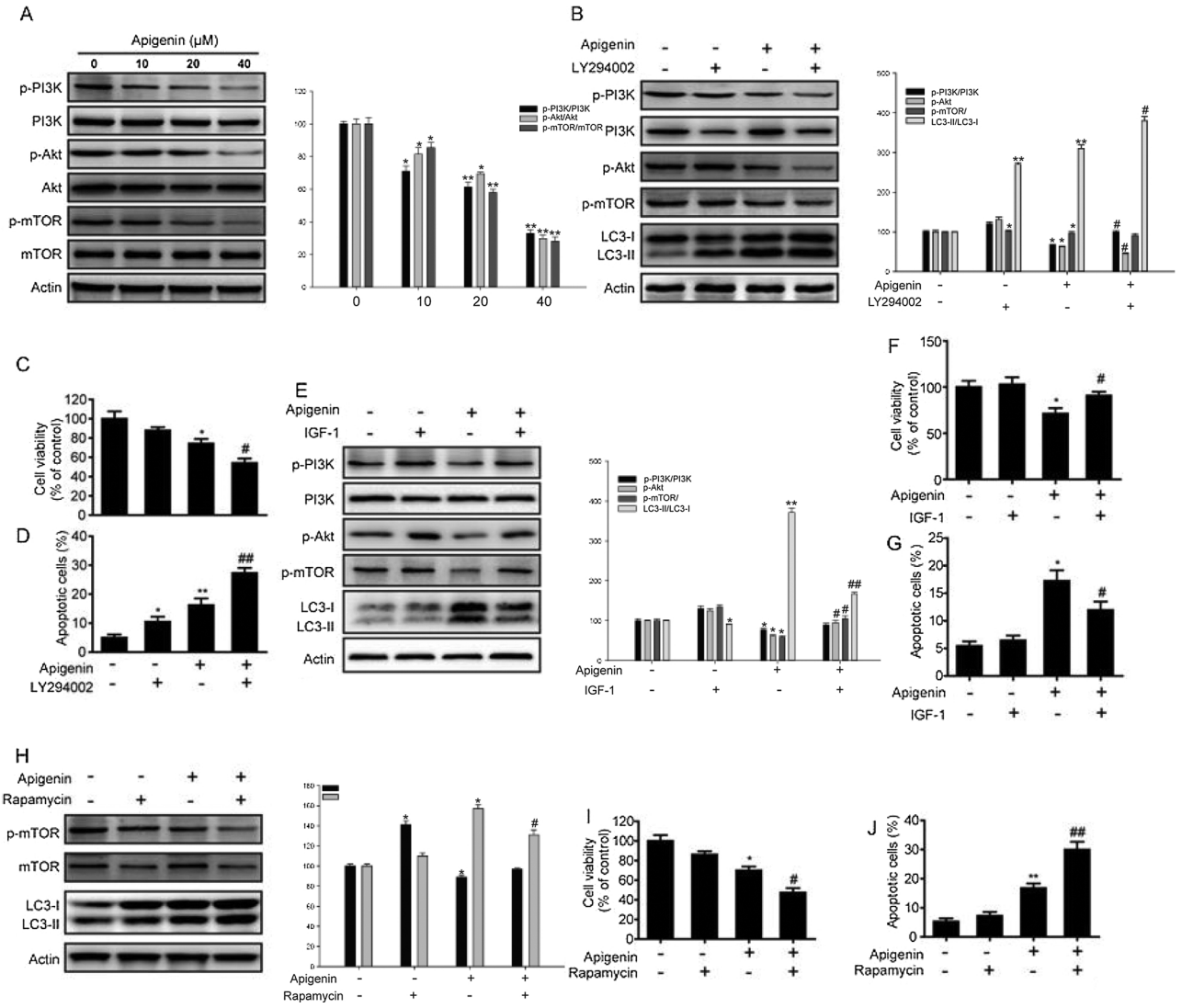


**Fig. 4. Apigenin induced apoptosis and autophagy via inhibition of the PI3 K/Akt/mTOR pathway in HepG2 cells.** (A) Cells were incubated with various concentrations (0, 10, 20 and 40 μM) of apigenin for 12 h. The protein levels of p-PI3K, PI3K, p-Akt, Akt, p-mTOR and mTOR were determined by Western blot. Cells were pretreated with LY294002 (10 μM) for 2 h and then incubated with apigenin (20 μM) for 12 h. (B) Cell lysates were subjected to western blotting. Cell viability (C) and apoptosis (D) were determined. Cells were pretreated with IGF-1 (10 μM) for 2 h and then incubated with apigenin (20 μM) for 12 h. Protein expression (E), cell viability (F) and apoptosis (G) were determined. Cells were pretreated with rapamycin (100 nM) for 2 h and then incubated with apigenin (20 μM) for 12 h. Protein expression (H), cell viability (I) and apoptosis (J) were determined. Data are presented as the mean ± SEM of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$ versus the control group, # $p < 0.05$, ## $p < 0.01$ versus the apigenin group.

autophagy utilizing inhibitors or siRNA could significantly enhance apigenin-induced caspase-3 activity and the number of apoptotic cells in HepG2 cells. These results indicated that autophagy may play a protective role in maintaining cell survival and proliferation under the stress of apigenin. Although inhibition of autophagy reinforces the antitumor effect of apigenin in vitro, the effective in vivo is needed to investigate. In the present study, we established xenografts models and treated the mice with 3-MA, apigenin and the combination of 3-MA and apigenin. Our result indicated that tumor volume and weight were significantly decreased in apigenin treated group and co-treatment of 3-MA with apigenin resulted in significantly inhibited cell proliferation and decreased tumor volume and tumor weight. Ki-67 immunohistochemistry staining further confirmed these results. These findings suggest that combined treatment of apigenin and autophagy inhibitor may a potential strategy to enhance the anti-tumor efficacy of apigenin activity.

Previous studies have been shown that several key molecules and signaling pathway could regulate autophagy [42,43]. Among them, PI3K/Akt/mTOR signaling pathway is well studied, which has a pivotal role in the regulation cell proliferation, differentiation and survival under normal physiologic and pathophysiological processes [44,45]. Drugs that target the PI3K/Akt/mTOR signaling pathway have the potential to inhibit survival pathways and induce apoptosis in cancer cells [46]. In this study, we found that treatment of hepatocellular carcinoma cells with apigenin decreased the levels of phosphorylated Akt and mTOR. mTOR is a serine/threonine kinase that regulate various cell signal pathways and process, such as apoptosis and autophagy [47]. Downregulation of constitutively phosphorylated Akt or mTOR by specific inhibitor reinforced HepG2 cells sensitive to apigenin. These results suggest that the apigenin-induced apoptosis and autophagy via suppressing the PI3K/Akt/mTOR signaling pathway in HepG2 cells.

In conclusion, we demonstrated that apigenin inhibited cell proliferation, induced apoptosis and autophagy in vitro and in vivo. Inhibition of autophagy promotes apoptosis induced by apigenin in HepG2 cells and xenograft model. Moreover, our study is the first to show that apigenin induced apoptosis and autophagy via inhibiting the

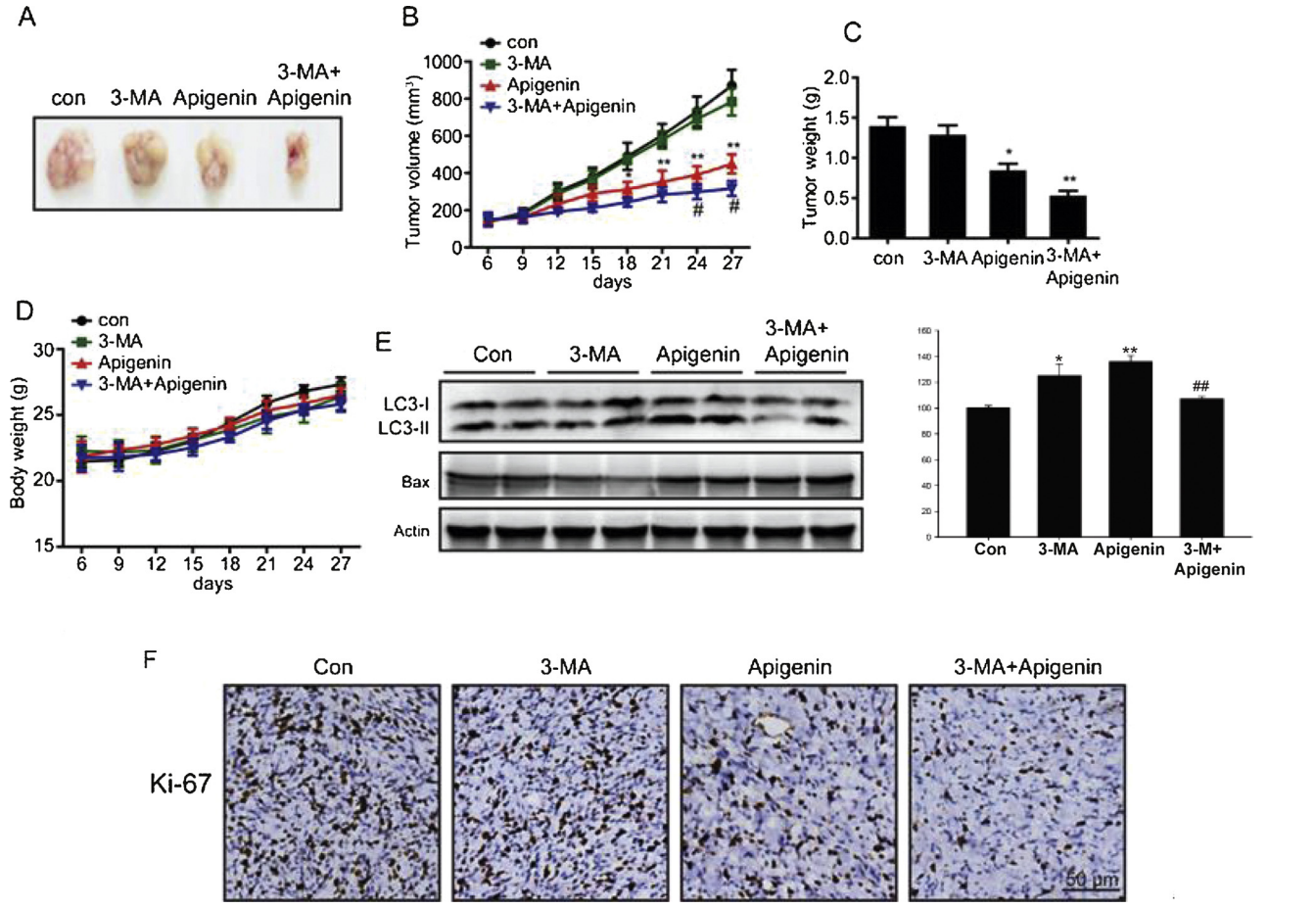


**Fig. 5. Inhibition of autophagy promoted apigenin-induced apoptosis in xenograft model.** HepG2 cells were injected subcutaneously into the lower right side flanks of 5 weeks old male BALB/c nude mice. After 6 days, animals were randomly divided into four groups; control, 3-MA (1 mg/kg), apigenin (100 mg/kg), and apigenin (100 mg/kg) combined with 3-MA (1 mg/kg). Mice were intraperitoneally injected with apigenin or 3-MA every 3 days. (A) Representative tumor images at the end of the treatment. (B) Average tumor volume was measured every 3 days. (C) Tumor weights were measured at the end of treatment. (D) Average body weight was measured every 3 days. (E) Western blot analysis on the expressions of LC3 and Bax from respective tumor tissues. (F) Ki-67 immunohistochemistry to detect proliferating cells in tumor sections. Values are expressed as mean ± SEM (n = 5). * $P < 0.05$, ** $p < 0.01$ compared to control mice; # $p < 0.05$, ## $p < 0.01$ compared to mice treated with apigenin.

PI3K/Akt/mTOR signaling pathway in hepatoma cells. All these findings suggest that offered us new insight into the anti-tumor activity of apigenin and helped us to understand the relationship between apoptosis and autophagy. Combined treatment of apigenin and autophagy inhibitor may a novel and effective therapeutic strategies to treat cancer.

## Conflict of interest

The authors state that they have no conflict of interests.

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