Sakurasosaponin inhibits lung cancer cell proliferation by inducing autophagy via AMPK activation

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Abstract. Sakurasosaponin (S-saponin; PubChem ID: 3085160), a recently identified saponin from the roots of *Primula sieboldii*, has shown potential anticancer properties against various types of cancer. In the present study, the effects of S-saponin on non-small cell lung cancer (NSCLC) cell proliferation and the underlying mechanisms, were investigated. The effect of S-saponin on cell proliferation and cell death were assessed CCK-8, clonogenic assay, western blotting and Annexin V/PI double staining. S-saponin-induced autophagy was determined by confocal microscopic analysis and immunoblotting. S-saponin inhibited the proliferation of A549 and H1299 NSCLC cell lines in a dose- and time-dependent manner, without inducing apoptosis. S-saponin treatment induced autophagy in these cells, as evidenced by the increased LC3-II levels and GFP-LC3 puncta formation. It activated the adenosine monophosphate-activated protein kinase (AMPK) signaling pathway, which is crucial for autophagy induction. Inhibition of AMPK with Compound C or siRNA-mediated knockdown of AMPK abrogated S-saponin-induced autophagy and partially rescued cell proliferation. Therefore, S-saponin exerts anti-proliferative effects on NSCLC cells through autophagy induction via AMPK activation. Understanding the molecular mechanisms underlying the anticancer effects of S-saponin in NSCLC cells could provide insights for the development of novel therapeutic strategies for NSCLC.

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Introduction

Lung cancer is a leading cause of cancer-related mortality [incidence ~2.2 million; mortality ~1.8 million (18%)] worldwide in 2020 and poses significant clinical and public health challenges (1). Despite advances in diagnostic techniques and therapeutic interventions, the prognosis of lung cancer remains poor, primarily because of late-stage diagnosis and the development of resistance to conventional therapies (2,3). Therefore, there is an urgent need to identify new therapeutic agents that can effectively inhibit lung cancer progression and overcome treatment resistance (4).

Adenosine monophosphate-activated protein kinase (AMPK) is a critical sensor of cellular energy status and plays a vital role in the regulation of cellular metabolism, growth and survival (5,6). AMPK is activated under conditions of metabolic stress, such as nutrient deprivation or hypoxia, and it functions to restore cellular energy homeostasis by promoting catabolic processes such as glucose uptake, fatty acid oxidation and autophagy (7-9). Activation of AMPK could also regulate the growth of various types of cancer, including lung, colorectal, and liver cancer, by inducing autophagy or mediating metabolic checkpoints (10,11). Therefore, AMPK activators are potential therapeutic candidates for cancer treatment. Inducing the activation of the AMPK signaling cascade using pharmacological or genetic strategies could exert potent anti-NSCLC cell activity (12-15). Accordingly, there is a need to develop novel AMPK activators with low toxicity and high efficiency to induce tumor cell inhibition. Autophagy is a cellular process that degrades and recycles unnecessary or damaged cellular components (16,17). It plays a crucial role in cell survival, differentiation, immune responses and cell death (16,18). In the context of cancer, autophagy plays a dual role, suppressing tumor growth by limiting the availability of nutrients and energy for rapidly dividing cancer cells or promoting tumor cell survival under stress conditions, leading to drug resistance and tumor relapse (19,20). Understanding the roles and mechanisms of autophagy and the related signaling pathways in cancer cells is important for targeting autophagy in cancer treatment.

Sakurasosaponin (S-saponin), a recently identified saponin present in a variety of plants [leaves of *Aegiceras corniculatum*, roots of *Jacquinia fammea Millsp* and *Primula sieboldii*

(P. sieboldii)], is a natural compound that exerts anticancer effects against different cancer types, such as breast, lung, colorectal, melanoma and prostate cancer (21,22). However, the molecular mechanisms underlying the anti-proliferative effects of S-saponin in lung cancer remain largely unexplored. In the present study, the main objective was to investigate the effects of S-saponin on the proliferation of the non-small cell lung cancer (NSCLC) cell lines, A549 and H1299 and to elucidate the underlying molecular mechanisms, with a focus on the roles of autophagy and AMPK signaling pathway. The results provide new insights into the molecular mechanisms underlying the anticancer effects of S-saponins and support the development of S-saponin-based therapies for NSCLC.

Materials and methods

Cell culture, antibodies and chemicals. The human NSCLC cell lines, A549 (cat. no. CCL-185) and H1299 (cat. no. CRL-5803) were obtained from the American Type Culture Collection. The cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Lonza Group, Ltd.) supplemented with 10% FBS (MilliporeSigma). S-saponin was kindly provided by Dr Nam-In Baek (Department of Oriental Medicine Biotechnology, Kyung Hee University, Yongin, Korea) (22). Antibodies against poly (adenosine diphosphate-ribose) polymerase (1:2,000; cat. no. 9545S, PARP), AMPK (1:3,000; cat. no. 2532S) and phosphorylated (p-)AMPK (1:3,000; cat. no. 2535S) were purchased from Cell Signaling Technology, Inc. Antibodies against GAPDH (1:5,000; cat. no. sc-47724) and tubulin (1:3,000; cat. no. sc-23948) were purchased from Santa Cruz Biotechnology, Inc. Horseradish-peroxidase-conjugated anti-mouse (1:2,000; cat. no. 31430) and horseradish-peroxidase-conjugated anti-rabbit secondary antibodies (1:2,000; cat. no. 31463) were purchased from Thermo Fisher Scientific, Inc.. Annexin V and propidium iodide (PI) were purchased from Molecular Probes; Thermo Fisher Scientific, Inc. Anti-LC3 II (1:2,000; cat. no. NB100-2220SS) antibody was purchased from Novus Biologicals, LLC. Compound C (cat. no. 171260) was purchased from MilliporeSigma.

Extraction and isolation of S-saponin. S-saponin was kindly provided by Dr Nam-In Baek (Department of Oriental Medicine Biotechnology, Kyung Hee University, Yongin, Korea) (22). Briefly, the air-dried roots of *P. sieboldii* (200 g) were extracted with 80% MeOH (3 L x3), and the concentrated extract (32.4 g) was poured in H_2O (500 ml) and extracted with EtOAc (500 ml x3) and n-BuOH (500 ml x3), successively. Each layer which was concentrated *in vacuo* gave the EtOAc fraction (PSE, 4.7 g), n-BuOH fraction (PSB, 12.8 g), and aqueous fraction (PSW, 14.9 g), respectively. The PSB was subjected to a silica gel (Kieselgel 60; Merck KGaA) column (8x15 cm) chromatography and eluted with $CHCl_3$-MeOH (10:1 → 5:1 → 3:1, 2l of each) and $CHCl_3$-MeOH-H_2O (10:3:1→7:3:1→65:35:10, 2 l of each) to produce 12 fractions (PSB1 to PSB12). Fraction PSB9 (8.4 g) was applied to octadecyl silica gel (ODS; LiChroprep RP-18, 40-60 lm; Merck KGaA) column (4x15 cm) chromatography and eluted MeOH-H_2O (3:2, 3.5 l) to afford 5 fractions (PSB1-1 to PSB-1-PBS-5) along with S-saponin at PSB-1-PBS-4 (7.1 g). The flow rate was 10 ml/min and temperature was 25˚C.

Cell proliferation assay. Cell viability was determined using a Cell Counting Kit-8 (CCK-8) assay kit (Dojindo Laboratories, Inc.) according to the manufacturer's instructions. Briefly, A549 and H1299 cells were seeded in 96-well plates at a density of $5x10^3$. After 24 h, the cells were treated with S-saponin at the doses or times indicated in the figures. CCK-8 solution (10/100 μl medium) was added to each well and the plate was incubated for 1 h in a CO_2 incubator at 37˚C. The absorbance of each well was measured at 450 nm using a microplate reader (Molecular Devices, LLC).

Clonogenic assay. The clonogenic assay was performed as previously described (22). Briefly, A549 and H1299 cells were seeded at equal densities in six-well plates ($1x10^3$ cells/well) for 24 h and then treated with S-saponin at doses of 0, 1, 2.5, 5, 7.5 and 10 μg/ml. The treated cells were cultured at 37˚C and 5% CO_2 for 7 days, and the colonies (≥50 cells) were fixed with 20% methanol for 10 min at room temperature and stained with 0.01% crystal violet for 10 min and counted using ImageJ (version 1.54f; National Institutes of Health).

Protein isolation and western blotting. Protein isolation and western blotting were performed as previously described (22). Briefly, A549 and H1299 cells were treated with S-saponin at the aforementioned doses and time intervals; subsequently, they were lysed in lysis buffer A [20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2 mM EGTA, 1% Triton X-100, 10% glycerol and protease inhibitor cocktail Set II (Sigma-Aldrich; Merck KGaA)], and centrifuged at 14,000 x g at 4˚C for 10 min. Cell lysates were collected after centrifugation and protein concentrations were determined using the Bradford method with Bio-Rad Protein Assay Kit II (Bio-Rad Laboratories, Inc.). The lysates (30 μg/lane) were separated through 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The samples were transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Inc.). After blocking with 5% milk in TBST (in 50 mM Tris, 200 mM NaCl, 0.2% Tween 20), the membrane was incubated in the first antibody solution diluted in 10% milk at 4˚C overnight. Subsequently, membranes were incubated with either horseradish-peroxidase-conjugated anti-mouse or anti rabbit secondary antibody for 1 h at room temperature. Proteins bands were visualized using ECL Western blotting reagents (GE-Life Science, Piscataway, NJ, USA). The results from western blot analysis were quantified using ImageJ (version 1.54f; National Institutes of Health).

Annexin V/PI staining. A549 and H1299 cells ($3x10^5$ cells/well) were treated with S-saponin for 24 h. Cell death was measured through fluorescence-activated cell sorting (FACS) using an Annexin V-fluorescein isothiocyanate/propidium iodide (Annexin V-FITC/PI) staining kit (cat. no. 556570; BD Biosciences). Fluorescence was measured using a fluorescence-activated cell sorting (FACS) Calibur (BD Biosciences), and the data were analyzed using CellQuest software (BD FACSDiva version 9.2; BD Biosciences).

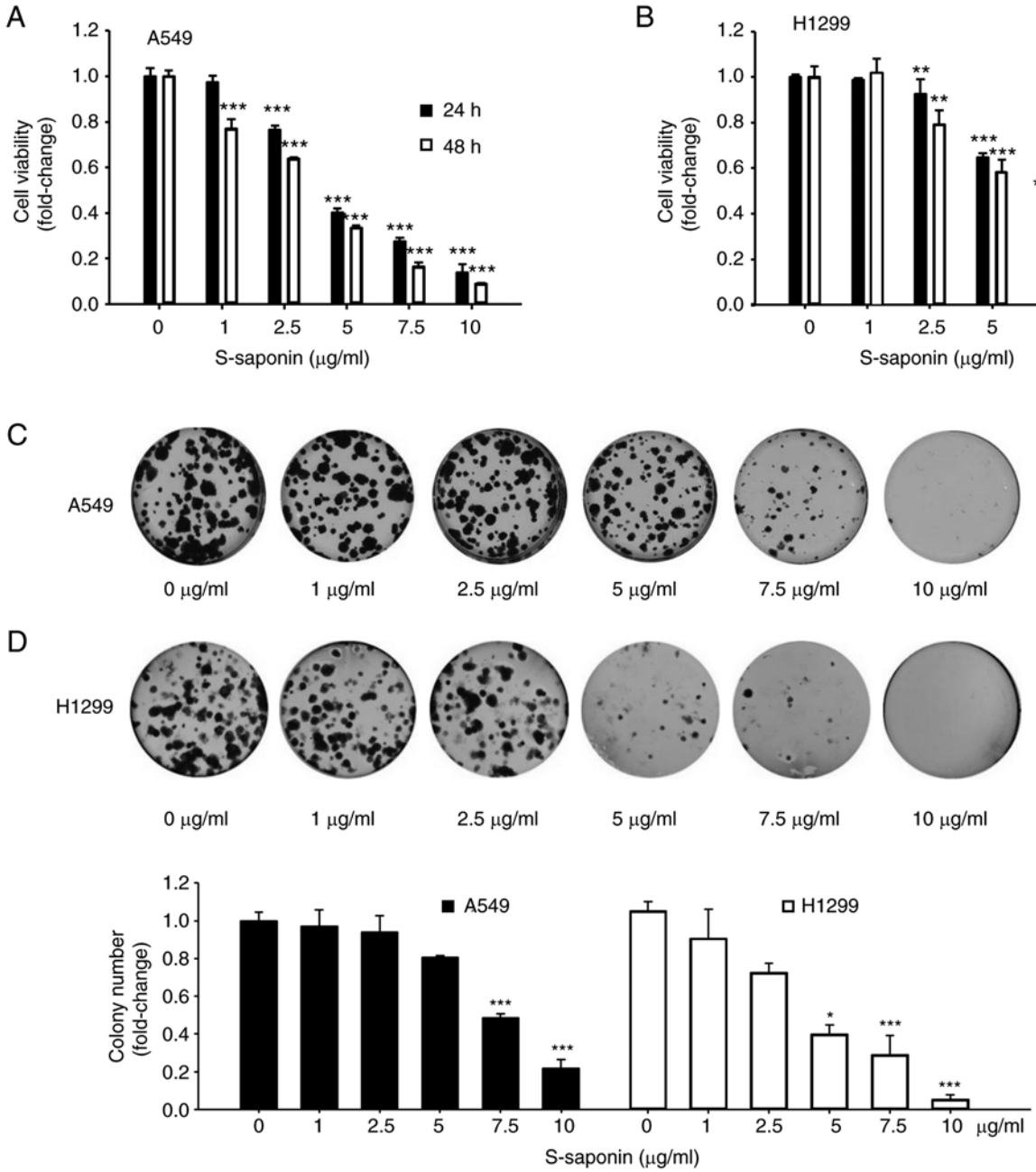

Figure 1. S-saponin inhibits non-small cell lung cancer cell proliferation. (A and B) A549 and H1299 cells were treated with various concentrations of S-saponin for 24 or 48 h, and cell proliferation was assessed using a Cell Counting Kit-8 cell viability assay. Data represent the mean ± SD of three independent experiments. (C and D) The clonogenic assay showing the effects of S-saponin on colony formation in A549 and H1299 cells. After treatment with S-saponin in a dose-dependent manner, the media was changed after 24 h. The media was then changed once every 2-3 days; the cells were fixed after 7 days of observation and counted following the staining with crystal violet. Representative images of colonies are shown, and quantitative data represent the mean ± SD of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. the control group. S-saponin, sakurasosaponin.

Immunofluorescence analysis. For LC3-II puncta detection, A549 and H1299 cells were transfected with GFP-LC3 vector (kindly provided by Dr Seung-Yong Yoon, Department of Brain Science, University of Ulsan College of Medicine, Seoul, Korea) and treated with or without S-saponin at 37˚C for 12 h. Cells were fixed with 4% paraformaldehyde in PBS at room temperature for 10 min. The cells were counterstained with Hoechst 33342 (cat. no. 62249, 1:1,000, Thermo Fisher Scientific) for 1 min at room temperature for nuclear staining. The coverslips containing the cells were then mounted with AquaMount (Lerner Laboratories; Thermo Fisher Scientific, Inc.) containing 0.01% 1,4-diazobicy clo(2,2,2)octane. Fluorescent images were obtained using a Leica confocal laser scanning microscope (Leica Microsystems GmbH).

Small-interfering (si)RNA and plasmid transfection. For AMPK knockdown, a siRNA against AMPKα (5'-AUG AUGUCAGAUGGUGAAUUU-3'; Bioneer Corporation) was constructed, exhibiting a specific knockdown efficiency of >90%. A549 and H1299 cells were transfected with the aforementioned specific siRNA or with a non-targeting siRNA (5'-UUCUUCGAACGUGUCACGU-3'; Bioneer

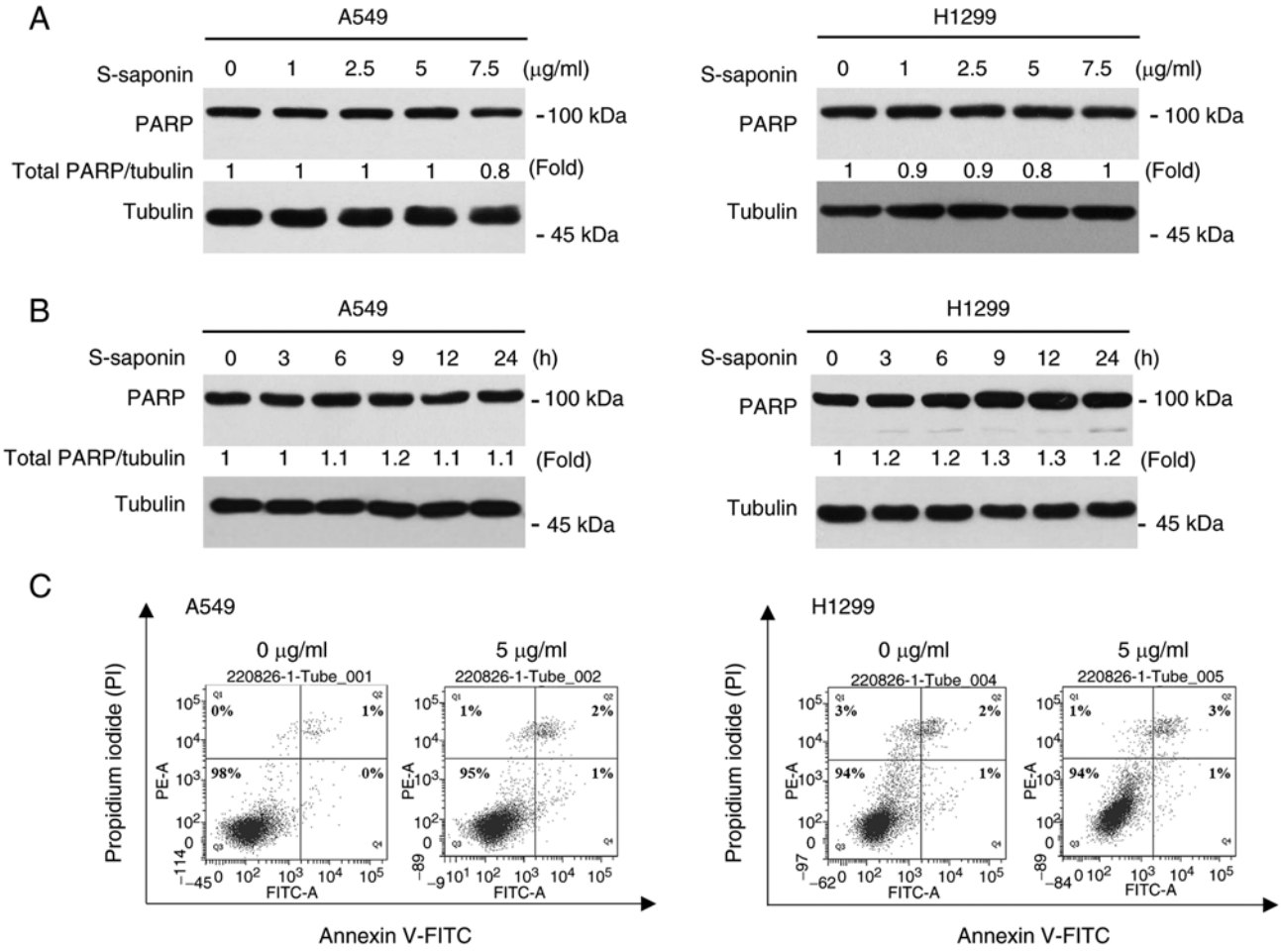

Figure 2. S-saponin does not induce apoptosis in A549 and H1299 cells. A549 and H1299 cells were treated with S-saponin (A) in a dose-dependent manner for 24 h and (B) in a time-dependent manner (5 μg/ml). The cells were subjected to western blotting using the PARP and tubulin antibodies. The experiment was independently performed thrice, quantified through the ImageJ software and normalized with tubulin. (C) A549 and H1299 cells were treated with S-saponin (5 μg/ml) for 24 h. The cell viability of S-saponin-treated A549 and H1299 cells was determined through FACS analysis using annexin V/PI staining. S-saponin, sakurasosaponin; PARP, poly (adenosine diphosphate-ribose) polymerase.

Corporation) at a final concentration of 50 nmol/l for 72 h using Lipofectamine® RNAimax (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols.

Statistical analysis. All the data in the present study are expressed as the mean ± standard deviation obtained from the results of three independent experiments. Statistical difference between multiple groups was analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's correction, using SigmaPlot 12.0 software (2013, Systat Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

S-saponin inhibits cell proliferation of NSCLC cells. To verify the anticancer effects of S-saponin on NSCLC cells, its effects were first examined on NSCLC cell proliferation. A549 and H1299 cells were incubated in growth medium containing various concentrations of S-saponin for 24 or 48 h, and their proliferation was determined using a CCK-8 cell viability assay. S-saponin treatment significantly inhibited the proliferation of A549 and H1299 cells in dose- and time-dependent manners (Fig. 1A and B). In addition, the clonogenic assay demonstrated that treatment with S-saponin markedly suppressed colony formation in the treated A549 (Fig. 1C) and H1299 (Fig. 1D) cells compared with that in the control cells. Next, it was examined whether apoptosis was involved in the effects of S-saponin on cell proliferation. Western blotting and Annexin V/PI double staining were used to assess the apoptotic rate in A549 and H1299 cells. PARP cleavage was assessed through western blotting to determine the status of apoptosis in S-saponin-treated A549 and H1299 cells in a time- and dose-dependent manner (Fig. 2A and B). S-saponin did not affect PARP cleavage. A549 and H1299 cells treated with S-saponin were subjected to Annexin V/PI double staining; S-saponin did not induce significant cell death (Fig. 2C). Therefore, it was concluded that the inhibitory effect of S-saponin on cell proliferation does not involve apoptosis.

S-saponin induces autophagy in NSCLC cells. S-saponin was extensively investigated in order to understand if autophagy activation is involved in the anti-proliferative effect of S-saponin in A549 and H1299 cells. The conversion of LC3 I to LC3 II and its subsequent translocation to autophagic vacuoles during autophagy induction is a hallmark of mammalian

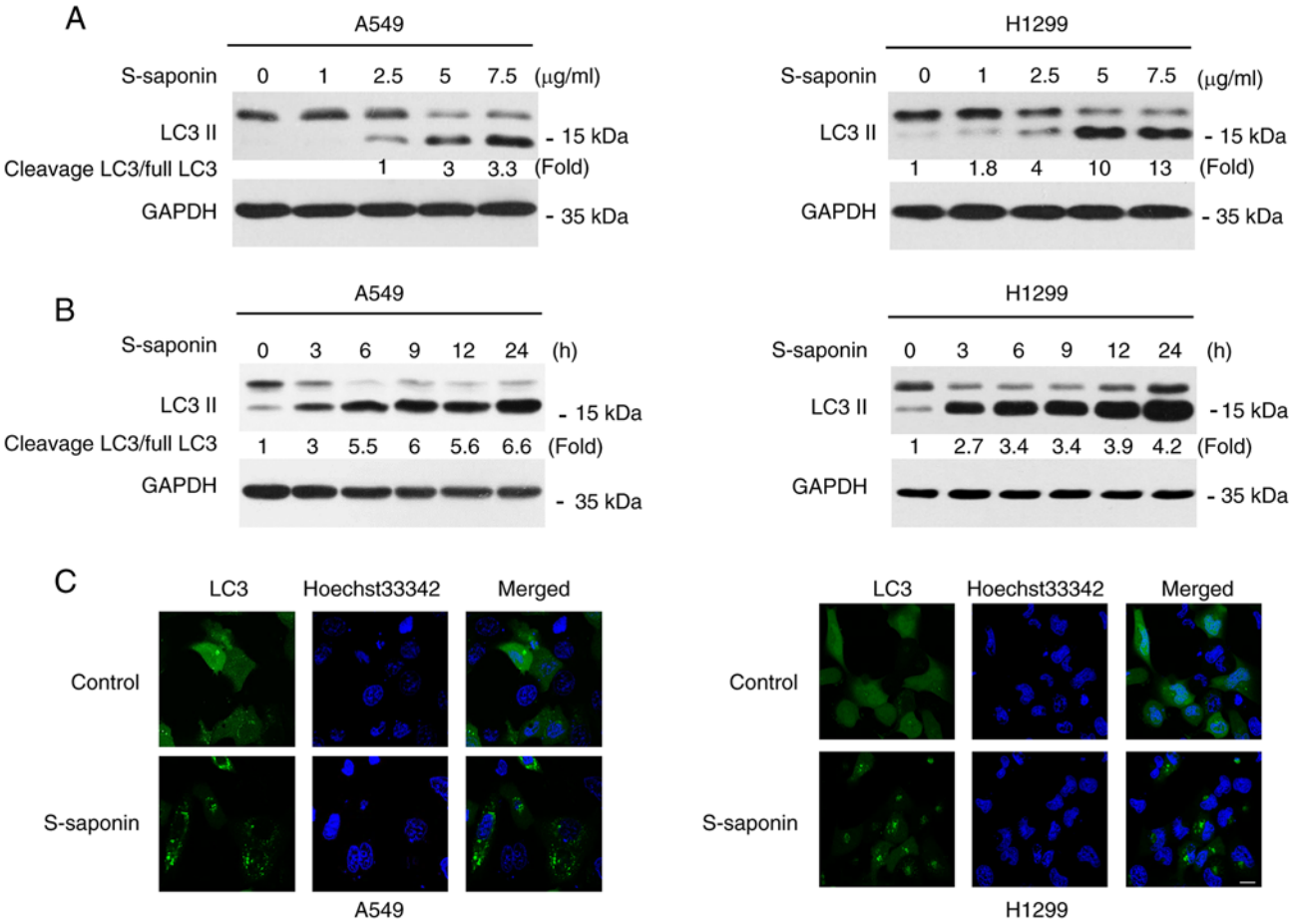

Figure 3. S-saponin induces autophagy in non-small cell lung cancer cells. A549 and H1299 cells were treated with S-saponin (A) in a dose-dependent manner for 24 h and (B) in a time-dependent manner (5 μg/ml). The cells were subjected to western blotting using the indicated antibodies. The experiment was independently performed thrice, quantified through the ImageJ software and normalized with full LC3 band. (C) A549 and H1299 cells were transfected with GFP-LC3 vector and treated with or without S-saponin for 12 h. After 12 h of culture, the cells were counterstained with Hoechst 33342. Fluorescence microscopy images of GFP-LC3 puncta formation were recorded using a Leica Confocal laser scanning microscope. Scale bar, 10 μm; magnification, x40. S-saponin, sakurasosaponin.

autophagy (23). A549 and H1299 cells were treated with different doses of S-saponin for different time periods, and the cell lysates were prepared and subjected to western blotting. Dose- and time-dependent increases in LC3 II levels following S-saponin treatment were observed in both cell lines (Fig. 3A and B). To further confirm S-saponin-induced autophagy, A549 and H1299 cells were transfected with a green fluorescent protein (GFP)-LC3 vector for 12 h followed by treatment with or without 5 μg/ml S-saponin. After 12 h, the distribution of LC3 was observed using a fluorescence microscope. S-saponin treatment significantly induced the formation of GFP-LC3 puncta in both A549 and H460 cells (Fig. 3C).

S-saponin influenced the AMPK phosphorylation pathway in NSCLC cells. AMPK is a sensor of cellular energy status and is activated under high intracellular AMP conditions, such as hypoxia or nutrient deprivation, inducing autophagy (9). To examine whether S-saponin phosphorylated AMPK in A549 and H1299 cells, the activation of AMPKα by S-saponin in the NSCLC cells was determined. A549 and H1299 cells were treated with different doses of S-saponin for different time periods, and the cell lysates were analyzed using western blotting. P-AMPKα was upregulated by S-saponin in a dose- and time-dependent manner (Fig. 4A and B).

AMPK is critical for autophagy-mediated cell proliferation of S-saponin-treated NSCLC cells. To further prove that S-saponin activates the AMPK signaling pathway, A549 and H1299 cells were incubated with 20 μM Compound C to block the AMPK signaling pathway before treatment with S-saponin. Western blotting results showed that Compound C inhibited AMPK activation by S-saponin and abolished the increase in LC3 II protein levels in S-saponin-treated cells (Fig. 5A). In addition, when A549 and H1299 cells were treated with 5 μg/ml S-saponin in the presence or absence of 20 μM Compound C for 18 h, the decrease in the proliferation of A549 and H1299 cells induced by S-saponin was significantly rescued (Fig. 5B). To illustrate the essential role of the AMPK signaling pathway in the autophagy-inducing effect of S-saponin, siRNA-mediated knockdown of AMPKα was utilized, an indispensable catalytic subunit of AMPK, to block the AMPK pathway. siRNA-AMPKα-transfected cells displayed a reduction in AMPKα expression, which blocked the upregulation of LC3 II by S-saponin (Fig. 6A). In addition, the clonogenic assay demonstrated that the colony numbers

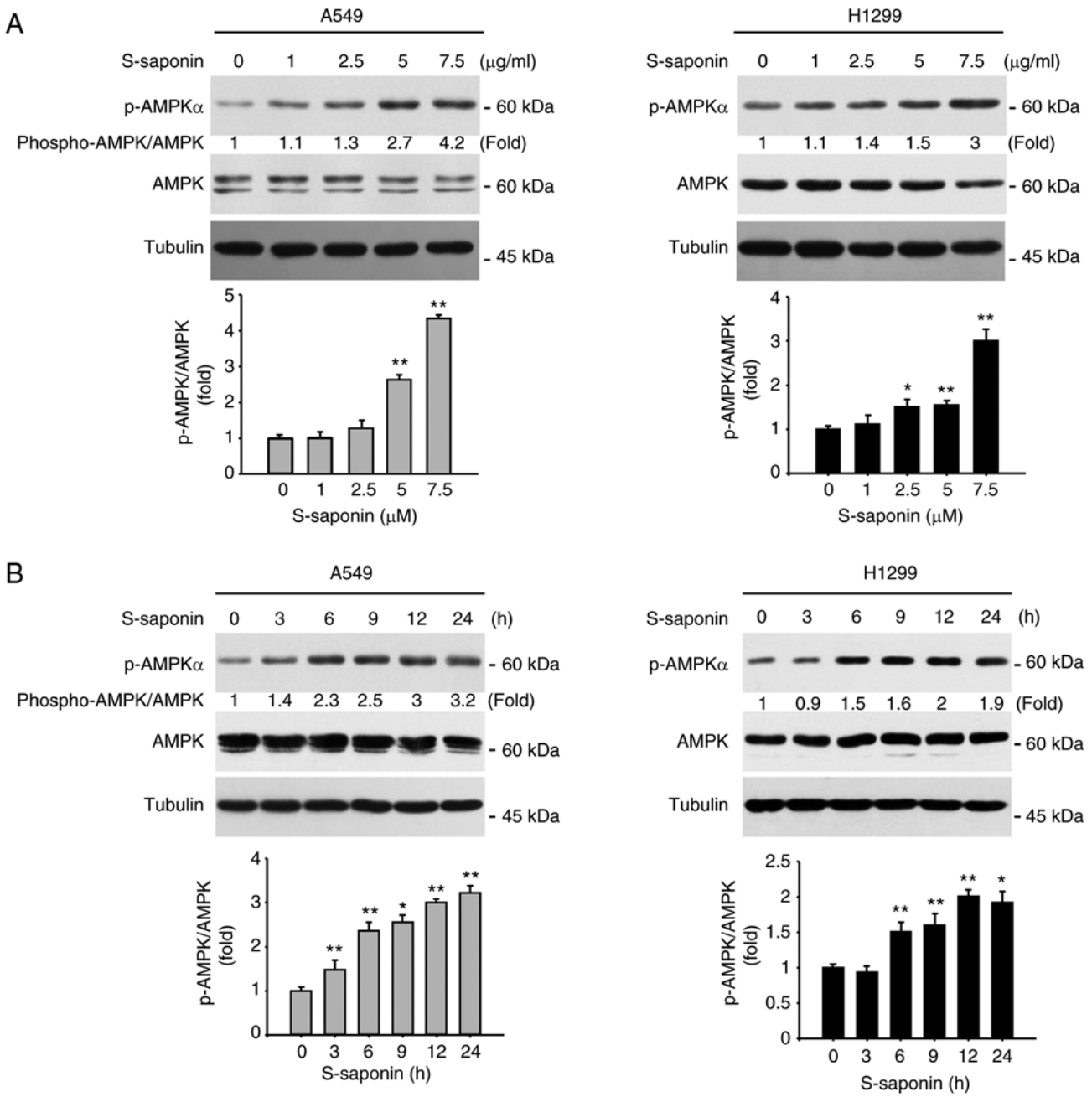

Figure 4. S-saponin activates the AMPK signaling pathway in A549 and H1299 non-small cell lung cancer cells. A549 and H1299 cells were treated with S-saponin (A) in a dose-dependent manner for 24 h and (B) in a time-dependent manner (5 μg/ml). The cells were subjected to western blotting using the phosphor-AMPKα and AMPKα antibodies. Tubulin was used as the loading control. The experiment was independently performed thrice, quantified through the ImageJ software and normalized with AMPK. *P<0.05 and **P<0.01 vs. 0 μM or 0 h. S-saponin, sakurasosaponin; AMPK, adenosine monophosphate-activated protein kinase.

were significantly rescued in the siRNA-AMPKα-transfected A549 and H1299 cells following S-saponin treatment (Fig. 6B).

Discussion

Lung cancer is the leading cause of cancer-related deaths worldwide, with NSCLC accounting for ~85% of all lung cancer cases (24). Despite advancements in diagnostic and treatment modalities, the overall survival rate of lung cancer patients remains low, emphasizing the need for new therapeutic strategies (2). In this context, the present study contributes to the growing body of research investigating the potential of natural compounds, such as S-saponin, in the treatment of NSCLC.

Natural products have long been considered rich sources of bioactive compounds with potential therapeutic applications in various diseases, including cancer (25-27). One such compound is saponin, a bioactive component found in various plants (28). Saponin possesses multiple pharmacological properties, including anti-inflammatory, anti-fibrotic, antioxidant and anticancer effects (29,30). The anticancer potential of saponins have been demonstrated in various types of cancers, including breast, gastric and colorectal cancers (31). However, the effects of S-saponin, a newly

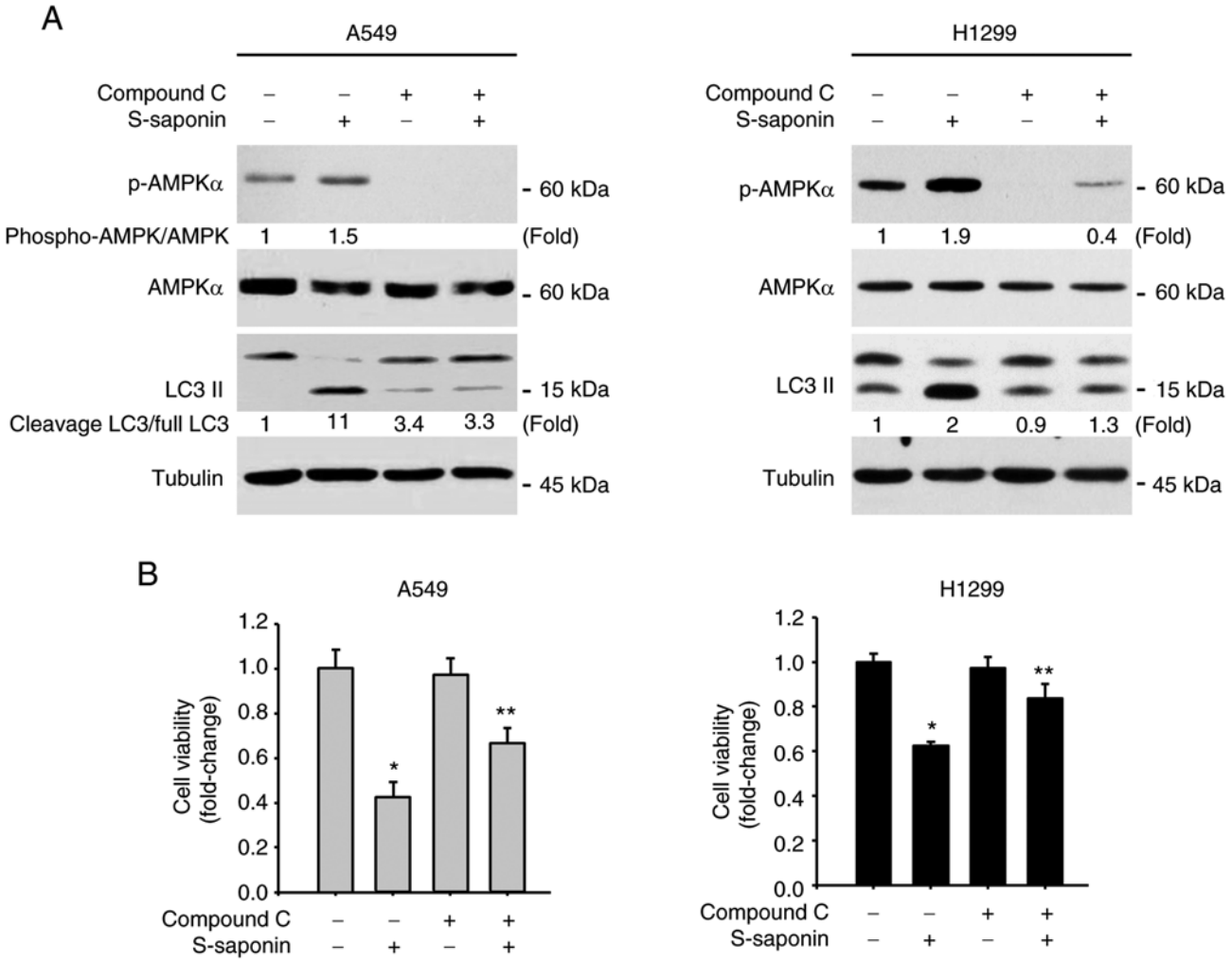

Figure 5. Pharmacologic inhibition of the AMPK pathway attenuates S-saponin-induced autophagy and cell proliferation inhibition. A549 and H1299 cells were pretreated with 20 μM Compound C for 30 min, followed by 5 μg/ml S-saponin for 24 h. (A) The cells were subjected to western blotting using p-AMPKα, AMPKα, and LC3 II antibodies. Tubulin was used as the loading control. The experiment was independently performed thrice, quantified through the ImageJ software and normalized with AMPK or full LC3 band. (B) A549 and H1299 cells were pretreated with 20 μM Compound C for 30 min, followed by 5 μg/ml S-saponin for 24 h. Cell proliferation was assessed using a Cell Counting Kit-8 cell viability assay. Data represent the mean ± SD of three independent experiments. *P<0.05 and **P<0.001 compared with the control group. AMPK, adenosine monophosphate-activated protein kinase; S-saponin, sakurasosaponin; p-, phosphorylated.

identified saponin from the root of *P. sieboldii*, on NSCLC cells and the underlying molecular mechanisms have not been fully elucidated.

S-saponin inhibited the proliferation of A549 and H1299 NSCLC cells in a dose- and time-dependent manner. This effect on cell proliferation was not mediated by apoptosis, as evidenced by the lack of PARP cleavage and Annexin V/PI double staining. Instead, S-saponin induces autophagy in NSCLC cells, as demonstrated by the dose- and time-dependent increases in LC3 II levels and GFP-LC3 puncta formation. These results are consistent with those of previous studies, highlighting the role of autophagy in the anticancer effects of various natural compounds (32).

Blocking the AMPK signaling pathway, either by using the AMPK inhibitor Compound C or siRNA-mediated knockdown of AMPKα, significantly rescued the inhibition of cell proliferation and induction of autophagy by S-saponin. These results emphasize the critical role of AMPK activation in the autophagy-mediated antiproliferative effects of S-saponin in NSCLC cells. The results of the present study are in accordance with a study by Liu *et al* (33), which reported that gitogenin, a saponin isolated from *Tribulus longipetalus*, induces autophagy in lung cancer cells via the AMPK signaling pathway. Similarly, a study by Xiang *et al* (34) demonstrated that Paris saponin VII, a bioactive constituent extracted from *Trillum tschonoskii* Maxim., inhibits the growth of NSCLC cells by inducing autophagy via the AMPK/mTOR pathway (34). These studies, together with results that were revealed in the present study, provide strong evidence for the involvement of AMPK-mediated autophagy in the anticancer effects of saponins in NSCLC cells.

In conclusion, the present study provides new insights into the molecular mechanisms underlying the anticancer effects of S-saponin in NSCLC cells, showing that S-saponin inhibits cell proliferation through the induction of autophagy via the activation of the AMPK signaling pathway. These findings contribute to the understanding of the potential therapeutic

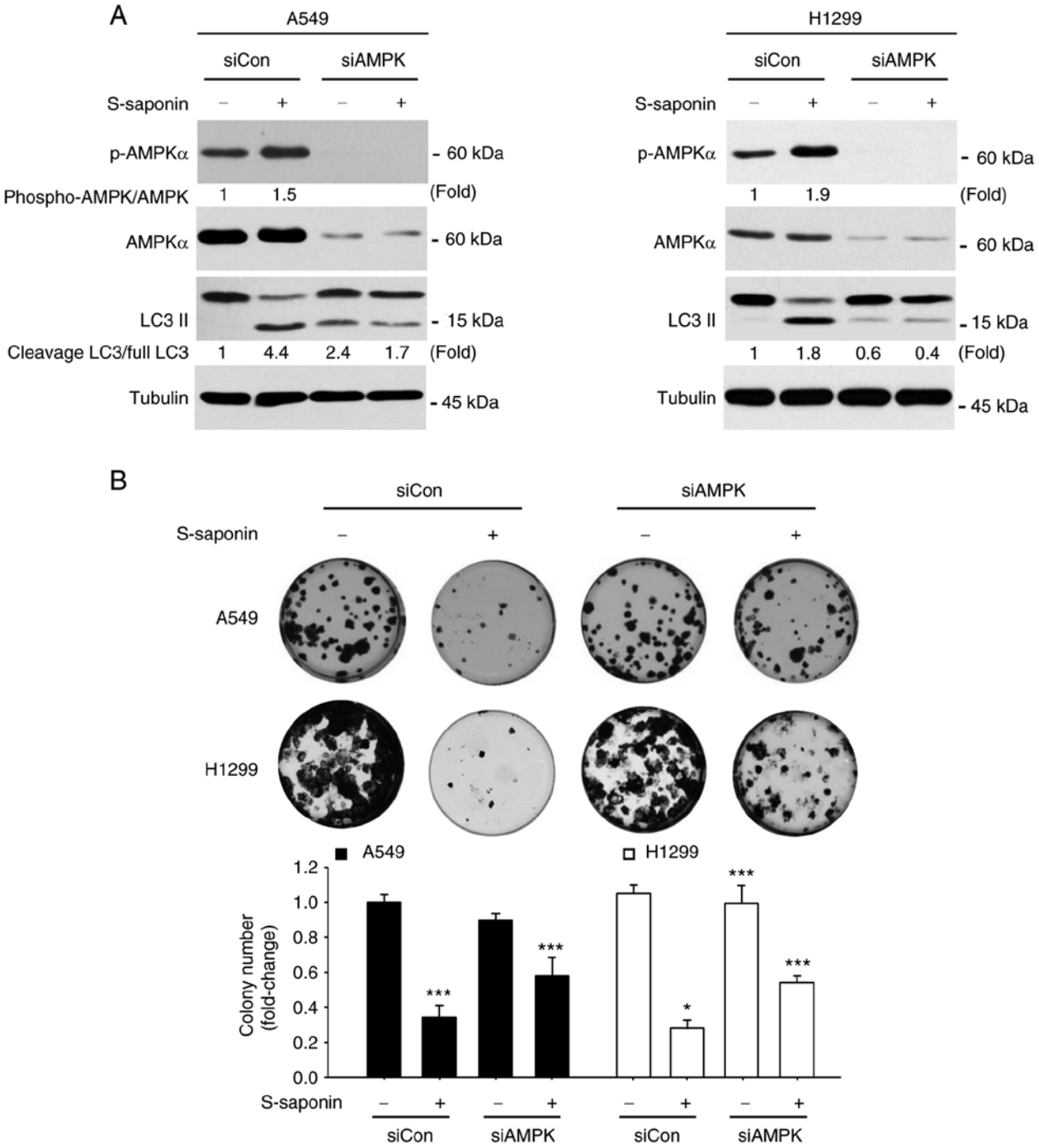

Figure 6. Knockdown of AMPK attenuates S-saponin-induced autophagy and cell proliferation inhibition. (A) A549 and H1299 cells were transfected with control siRNA or siRNA-AMPKα and treated with or without 5 μg/ml S-saponin. The cells were subjected to western blotting using p-AMPKα, AMPKα, and LC3 II antibodies. Tubulin was used as the loading control. The experiment was independently performed thrice, quantified through the ImageJ software and normalized with AMPK or full LC3 band. (B) A549 and H1299 cells were transfected with control siRNA or siRNA-AMPK and treated with or without 5 μg/ml S-saponin. Following the treatment with S-saponin, the media was changed once after 24 h, and then once every 2 days; the cells were fixed after 4 days of observation and counted following the staining with crystal violet. Representative images of colonies are shown, and quantitative data represent the mean ± SD of three independent experiments. *P<0.05 and ***P<0.001 compared with control group. AMPK, adenosine monophosphate-activated protein kinase; S-saponin, sakurasosaponin; si, small interfering; p-, phosphorylated.

applications of S-saponin in NSCLC treatment and pave the way for future research on its efficacy in combination therapies and in *in vivo* models.

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Availability of data and materials

All data generated and analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YS, CL and SJ designed the present study. YS, CL, JL, JK, YK and PL performed experiments. YS, CL and SJ analyzed the data. YS and SJ wrote the manuscript. YS, JL, JK, YK, PL and SJ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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