Salicylic Acid Inhibits Growth and Sensitizes Cervical Cancer Cells to Radiotherapy by Activating AMPK/TSC2/mTOR Pathway

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Research

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Abstract

Background

Radioresistance remains a major clinical challenge in cervical cancer therapy. Salicylic acid (SA)-mediated direct activation of AMP–activated protein kinase (AMPK) is critical to radiosensitivity. However, limited data exists regarding the combination of SA and radiotherapy, even though there are several indications that this might be a promising treatment strategy. This study aimed to investigate the radiosensitizing effect of SA on human cervical cancer cells and its potential molecular mechanism.

Methods

Cervical cancer cells were treated with SA and ionizing radiation. The expression of γ-H2AX was evaluated by immunofluorescence (IF) assay. Cell cycle and apoptosis were analyzed by flow cytometry. Western blot was performed to detect the protein level of AMPK/TSC2/mTOR pathway.

Results

SA inhibited basal proliferation of cervical cancer cells in a dose and time dependent manner. In addition, SA increased radiation-induced DNA damage, promoted apoptosis, triggered a redistribution of cell cycle from G2-M phase to G1-S phase of cervical cancer cells, and hence increased cell sensitivity to radiation. Moreover, SA treatment elevated the expression levels of p-AMPKα and p-TSC2, whereas the level of p-mTOR was significantly decreased.

Conclusion

SA enhances the radiosensitivity of cervical cancer cells by targeting AMPK/TSC2/mTOR signaling pathway, and might serve as a promising therapeutic strategy to improve the efficacy of radiotherapy for cervical cancer.

Introduction

Cervical cancer is the fourth most commonly diagnosed cancer and a significant cause of cancer(1). The standard of care for treatment of advanced cervical cancer is the combination of concurrent chemotherapy with external beam radiation therapy (EBRT) followed by an intracavitary brachytherapy (ICBT) boost(2). This comprehensive treatment achieves a favorable outcome for cervical cancer patients,however, there are still over 13% of patients suffering local recurrence following radical radiotherapy due to radioresistance(3), indicated the importance for developing novel strategies to improve the sensitivity of cervical cancer to ionizing radiation (IR).

Salicylic acid (SA), the primary metabolite of Aspirin, is quite an "old medicine" widely used for pain, fever, and inflammation since ancient Greece. Its mechanisms of action have been proposed, including inhibition of cyclooxygenase(4), IKK-β activity(5),topoisomerase II(6) as well as NF-kB(7).Moreover, in 2012, SCIENCE reported that SA could directly bind to and activate AMP-activated protein kinase (AMPK) without relying on AMPK upstream kinase(8).AMPK is a critical cellular energy sensor that has well-known roles in inhibiting cancer growth(9).Notably, growing evidence suggests that the activation of AMPK effectively enhances the radiation response of multiple cancer types and may serve as a positive regulator of radiosensitivity(10–12).We wonder if SA-mediated direct activation of AMPK may sensitize cervical cancer cells to radiation. Recently, the anti-tumor activity of SA has been reported in several pieces of researches(13–16); however, few studies focus on the radiosensitizing role of SA in cancer treatment.

Here, we evaluated the effects of SA on human cervical cancer cell proliferation and radiosensitivity. We also further identified the underlying molecular mechanisms. Our findings may provide novel insight into the contribution of the well-documented drug to cancer treatment and identify SA as a promising therapeutic approach to improve the efficacy of radiotherapy for cervical cancer.

Material And Methods

Cell cultures and treatment

Human cervical cancer cell line CaSki and Hela were obtained from Cell Bank of Shanghai Institute for Biological Science and were cultured in Rosewell Park Memorial Institute (RPMI) 1640 medium (Gibco) containing 10% fetal bovine serum (Gibco) and 1% penicillin streptomycin (Beyotime Biotechnology). All cell lines were incubated at 37 °C in the presence of 5% CO2. Salicylic acid (SA, Sigma) was dissolved in DMSO to a concentration of 4 mmol/L and stored at -20˚C for up to 4 weeks. For the hypoxic treatment, cells in exponential phase of growth were incubated in culture media with 100μm/l cobalt chloride (CoCl2), a commonly mimetic hypoxia reagent.

Cell proliferation assay

Cells were seeded in 96-well plates at a density of 5000 cells per well and allowed to adhere overnight. After treatment with various SA concentrations as indicated, cells were washed with phosphate-buffered saline (PBS), fixed with methanol, and stained with 0.5% crystal violet dye. After drying, the crystal violet was next solubilized in 1% sodium dodecyl sulfate solution (SDS). The absorbance density was recorded at 570 nm using a microplate reader (BioTek, USA). IC20 was determined to be a treatment concentration that depressed cell proliferation by 20%.

Colony formation assay

CaSki and Hela cells were seeded in 6-well plates overnight. After pretreating with SA for 1 h, cells were irradiated by X-ray linear accelerator (Varian, dose rate: 200cGy/min; ray energy: 6MV).For hypoxia

treatment, Caski cells were treated with 100μm/l $CoCl_2$ for 4 h, SA were added into medium 1h before radiation treatment. The medium containing SA was then removed, and cells were cultured in a standard medium to form colonies. Fourteen days later, the cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The colonies containing more than 50 cells were counted under a microscope.

Immunofluorescence staining

Cells were seeded into 24-well plates overnight and then treated with or without SA for 1 h before radiation exposure (8 Gy). After the irradiation, cells were fixed with 4% paraformaldehyde at predetermined time points, followed by permeabilizing with 0.2% Triton X-100. Subsequently, the cells were stained with the anti-γ-H2AX antibody (1:500 dilution; Abcam) at 4 °C overnight and then with a goat anti-rabbit IgG fluorescent-conjugated secondary antibody (1:200 dilution; Beyotime Biotechnology) for 30 min at 37 °C. The nuclei were counterstained with DAPI. The images of the γ-H2AX foci were observed with an Olympus confocal microscope.

Apoptosis and cell cycle assay

Apoptosis was detected with the KGI Biotechnology Apoptosis Kit according to the manufacturer's protocol. For cell cycle assay, cells were collected and fixed with 70% ethanol and then stained with propidium iodide (PI, Biolegend). Flow cytometry was performed using a BD FACSCalibur flow cytometer (BD Biosciences, USA). Data were analyzed using the FlowJo software.

Western Blotting

Total protein was extracted with RIPA supplemented with 1% PMSF (Beyotime Biotechnology). We used a BCA kit (Beyotime Biotechnology) to test the protein concentrations. Equal amounts of protein samples were separated in SDS-PAGE gels and transferred onto PVDF membranes (Millipore, CA, USA). After blocking with 5% nonfat milk, the membranes were incubated with primary antibodies at 4°C overnight against phosphor-AMPKα (Thr172) (1:800 dilution; Cell Signaling Technology), AMPKα (1:1000 dilution; Abcam), β-actin (1:2000 dilution; Proteintech), phospho-TSC2 (Ser1387) (1:500 dilution; Cell Signaling Technology), TSC2 (1:2000 dilution; Cell Signaling Technology), phospho-mTOR (1:500 dilution; Abcam), mTOR (1:1000 dilution; Abcam). Next, membranes were washed and incubated with HRP-conjugated secondary antibodies (1:5000 dilution; Beyotime Biotechnology) at room temperature for 1 h. Images were visualized with a chemiluminescence detection system. The gray values of the western blotting bands were procured using Image J software.

Statistical analysis

All experiments were independently performed three times. Data are presented as the mean ± (SD) and were statistically analyzed using GraphPad Prism software version 8.01 (GraphPad Software, San Diego, CA). Differences between groups were evaluated with an unpaired two-tailed Student's *t*-test or analysis of variance (ANOVA) followed by Bonferroni post-test. A *P*-value < 0.05 was considered statistically significant.

Results

SA inhibits cervical cancer cell growth and sensitizes cells to IR

Although SA was previously implicated in cancer cell proliferation suppression, its radiosensitive activity on cervical cancer cells has not been characterized. To address this issue, we chose the 2 cervical cancer cell lines, CaSki, and Hela, as our cellular models and first tested the effect of SA on cervical cancer cell proliferation. As presented in Figure 1A, CaSki cells were treated with various concentrations (ranging from 0.5-10mmol/L) of SA for 24, 48, and 72 hours. The results showed a gradually decreased cell viability rate of CaSki cells, accompanied by gradually increased SA concentration and action time. Similar findings were observed by Hela cells (Fig 1B), suggesting that SA inhibits cervical cancer cell growth in a dose and time-dependent manner.

Given the potentially important role of SA-mediated direct activation of AMPK in radiosensitivity, we also investigated whether SA changes the radiosensitivity of cervical cancer cells to IR. IC20 was a commonly used concentration when evaluating the effect of a drug on radiation sensitization(17, 18), we thus chose the IC20 of SA (4mM) for the following experiment. To understand SA action time course, we treated CaSki cells with 4mM SA for 0.5–24 h; and observed obvious evidence of AMPK phosphorylation (Thr172) within 0.5 h, which reached the highest level by 1 h (Fig 1C). Accordingly, we next challenged CaSki and Hela cells with 4mM SA for 1h followed by IR with 0, 2, 4, 6, 8 Gy. There was a significant reduction in the SA plus IR group's clonogenic survival compared with IR alone both in CaSki and Hela cells (Fig 1D-F). Together, these results demonstrate that SA effectively enhances radiation sensitization of human cervical cancer cells.

SA impairs cell repair of radiation-induced DNA double-strand break

IR triggers cellular apoptosis by inducing DNA damage; we, therefore, wondered whether SA promotes IR-induced DNA double-strand breaks (DSBs). To this end, we measured γ-H2AX foci formation by immunofluorescence staining after irradiation. γ-H2AX foci is a sensitive marker of DSBs and often be used to monitor DNA repair(19, 20). As shown in Figure 2A, SA alone did not appear to affect DNA repair because the percentage of γ-H2AX positive cells was very low and unaffected upon SA treatment. However, when combined with radiation treatment, a significant increase in γ-H2AX foci was observed in SA plus IR group at 2 h from radiation treatment (Fig 2B). Notably, such an enhancement still persisted at 24 h after IR, indicating that SA increases IR-induced DNA damage and prolongs DNA damage repair of cervical cancer cells (Fig 2C).

SA suppresses IR-mediated cell cycle arrest, whereas it facilitates IR-induced apoptosis

Cellular DNA damage activates cell cycle checkpoints, thereby alter cell cycle distribution(21).We next analyzed whether SA effect IR-mediated cell cycle alterations. Flow cytometric analysis indicated that SA

alone did not alter cell cycle distribution ($P > 0.05$). Upon irradiation, the population in G2/M phase was significantly enriched, presenting a G2/M arrest (control 13.05% *vs* IR 25.38%, $P < 0.05$). SA combined with IR caused accumulation of radiated cells into the G1 phase (IR 47.35% *vs* SA+IR 76.29%, $P < 0.05$), decreased the number of cells at relative radioresistant S-phase of cell cycle (IR 27.86% *vs* SA+IR 14.58%, $P < 0.05$), and to some extent counteracted IR-mediated G2/M arrest (IR 25.38% *vs* SA+IR 9.13%, $P < 0.05$) (Fig 3).

To further address the role of SA on IR-mediated apoptosis, we also examined the percentage of apoptotic cells by flow cytometric analysis. As expected, exposure of radiation enhanced the apoptosis rate of Caski cells. This increase was more pronounced in the combination therapy group, suggesting that SA also facilitates cervical cancer cell apoptosis induced by radiation (Fig 4).

SA combined with IR activates AMPK/TSC2/mTOR pathway

The above results indicated that SA suppresses IR-mediated DNA damage response and cell cycle arrest while promoting apoptosis, resulting in increased cervical cancer radiosensitivity. Next, we sought to investigate the underlying molecular mechanisms. Since activated AMPK inhibits protein synthesis, thereby inhibiting cell growth and proliferation; and promoting radiosensitivity mainly by regulating TSC2-mTOR pathway(22),we wonder if SA sensitizes cervical cancer cells to radiotherapy by regulating AMPK/TSC2/mTOR pathway. As shown in Figure 5, compared with the control group, the expression of p-AMPK was higher in either SA group or IR group, and SA combined with IR further increased the level of p-AMPK.

Activation of the p-TSC2 site promotes the formation of TSC2 and TSC1 complexes, thereby inhibiting mTOR phosphorylation. We found that the levels of TSC2 in SA group, irradiated group, and the combined group were higher than those in control group, and TSC2 level in SA combined group was significantly higher than that in radiation group. Furthermore, the expression of AMPK downstream signal molecule p-mTOR was significantly increased after irradiation, while the p-mTOR induced by radiation was significantly down-regulated after adding SA treatment. Overall, these data suggest that SA increases the radiosensitivity of cervical cancer cells, at least in part, by activating AMPK/TSC2/mTOR pathway (Fig 5).

Discussions

SA has been suggested antitumor properties in colorectal cancer, leukemia and mesothelioma(14–16); however, to date, few studies examine the effects of SA in cervical cancer models, not to mention its combination with radiotherapy, even though this may provide valuable clues to improve the efficacy of radiotherapy for cervical cancer. Here, we show significant antiproliferative and radiosensitizing effects of SA in human cervical cancer cells. SA has a well-known pharmacological effect in anti-inflammatory treatment(23),our findings broaden the potential clinical application of SA to the treatment of cervical cancer.

H2AX (phosphorylated derivative named γ-H2AX) was considered as a target for activating ataxia telangiectasia mutated (ATM), which induced by ionizing radiation through DSB(19–22).It plays an important role in the subsequent damage repair process(24),therefore, the formation of γ-H2AX focal points was usually used as a predictor for the degree of DNA DSBs. Consistent with previous literature, γ-H2AX foci rapidly increased after IR(25).SA treatment did not significantly affect DNA DSBs of resting CaSki cells. However obviously enlarged IR-induced DSBs, evidenced by increased γ-H2AX foci formation, such increasing remains significantly for more than 24 hours, providing direct evidence of increased radiation sensitivity.

Intriguingly, cycle and apoptosis experiments showed that SA alone did not significantly alter the cycle distribution of CaSki cells, while SA combined with radiation reduced the radiation induced G2/M blockade and significantly increased G1 cycle arrest. At the same time, the apoptosis rate of CaSki cells was increased significantly after treated with SA alone, we found such an increase was further enhanced when combined with radiation. The possible reason is that SA combined with radiation further increased AMPK activation, and significant inhibition of radiation induced p-mTOR expression; at this time, the increase of P53-P21 further increased AMPK mediated apoptosis and blocking P53 mediated G1 cycle.

As a receptor that perceiving the balance of cell energy metabolism, AMPK regulates the three major metabolisms of carbohydrate, fat and protein, and plays an important role in maintaining cell energy balance(26). Recent studies have shown that AMPK regulate a series of tumor suppressor genes, such as LKB1, P53 and TSC1/2, it also have a close relationship in the occurrence and development of malignant tumors(27–29). The activation of AMPK by inhibition of the mammalian target of rapamycin (mammalian target of rapamycin, mTOR) inhibit protein synthesis, cell cycle checkpoint activation such as the activation of p53 and cyclin dependent protein kinase (CDK) inhibitor p21cip1 blocked cell cycle progression, proliferation and inhibition from cells, and increase the radiosensitivity of cells(30). *In vitro* studies have found that the radiation sensitivity of cells decreased after the inhibition of AMPK. Using AMPK - / MEFs - alpha 1/2 or siRNA silencing AMPK, the survival rate of lung cancer cells was increased significantly after radiotherapy(10, 22, 31). On the contrary, resveratrol, metformin and other AMPK activators combined with radiation increased the cytotoxic effect on cancer cells, which suggested that the level of AMPK activation might influence the efficacy of radiotherapy to some extent. So far, few drugs was found directly activate AMPK, but often depend on AMPK upstream kinase LKB1(32, 33). In this study, we found that SA directly activates AMPK; whether it is SA treatment simply or radiotherapy alone. This finding is in line with Storozhuk Y(10). Combine the evidence above; we suggested SA may upregulate the radiosenstivity of cervical cancer cells by activation on AMPK.

MTOR have crucial functions in controlling cell growth and metabolism. It is found that mTOR mainly stimulates the PI3K/AKT pathway through growth factors, relieves the direct inhibition of TSC1/TSC2 complex on mTOR, thereby activating mTOR(34). Inhibition of mTOR specifically increase the radiosensitivity of cells(35). When cells are exposed to metabolic stress or lack of nutrition, AMPK can directly activate Ser1345 on TSC2, activate TSC2, form TSC1/TSC2 complex, thereby inhibiting mTOR activity(34). Previous studies have reported that irradiation stimulates cell growth stress signals, and

further activates AKT/mTOR, resulting in radioresistance. In this study, we also observed increased mTOR level upon irradiation. For this reason, we intend to suppress mTOR signal by further activating AMPK. We show TSC2-mTOR was activated rapidly after AMPK activation either in SA group, radiation group or combined group. Further analysis showed that SA induced the phosphorylation of p-AMPK Thr172 and p-TSC2 Ser1387, and significantly reduced IR-induced p-mTOR Ser2448. Although Ser1345 has been reported to activate TSC2(34), this study found that AMPK also phosphorylated TSC2 on the Ser1387 site to activate TSC2, thereby inhibiting mTOR, which was also verified in Hong-Brown's study(36, 37).

Conclusion

In summary, the current study demonstrates that SA significantly inhibits the proliferation of cervical cancer cells, impairs cell repair of radiation-induced DNA doublestrand break, suppresses IR-mediated cell cycle arrest, promotes IR-induced apoptosis, and hence increases cervical cancer cell sensitivity to radiation. Our data may provide a new clue for improving the radiation resistance and improving cervical cancer's therapeutic effect.

Abbreviations

SA, salicylic acid;

AMPK, AMP–activated protein kinase;

EBRT, external beam radiation therapy;

ICBT, intracavitary brachytherapy;

ANOVA, analysis of variance.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

The authors declare no conflict of interest.

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Authors contribution

Jie Zhou, Wenjing Ye and Guangming Yi contributed equally to this work and are co-first authors. Jie Zhou and Jianming Huang conceived and designed the study; Jie Zhou and Shun Lu wrote the paper; Wenjing Ye and Guangming Yi carried experiments; Siyao Deng and Yimin Li performed the data analysis; Yanqiong Song and Jiayu Zhang completed data curation and validation; MingLun Li and Lichun Wei reviewed and edited the paper; Jinyi Lang and GuiQuan Zhu provided resourses and funding.

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Figures

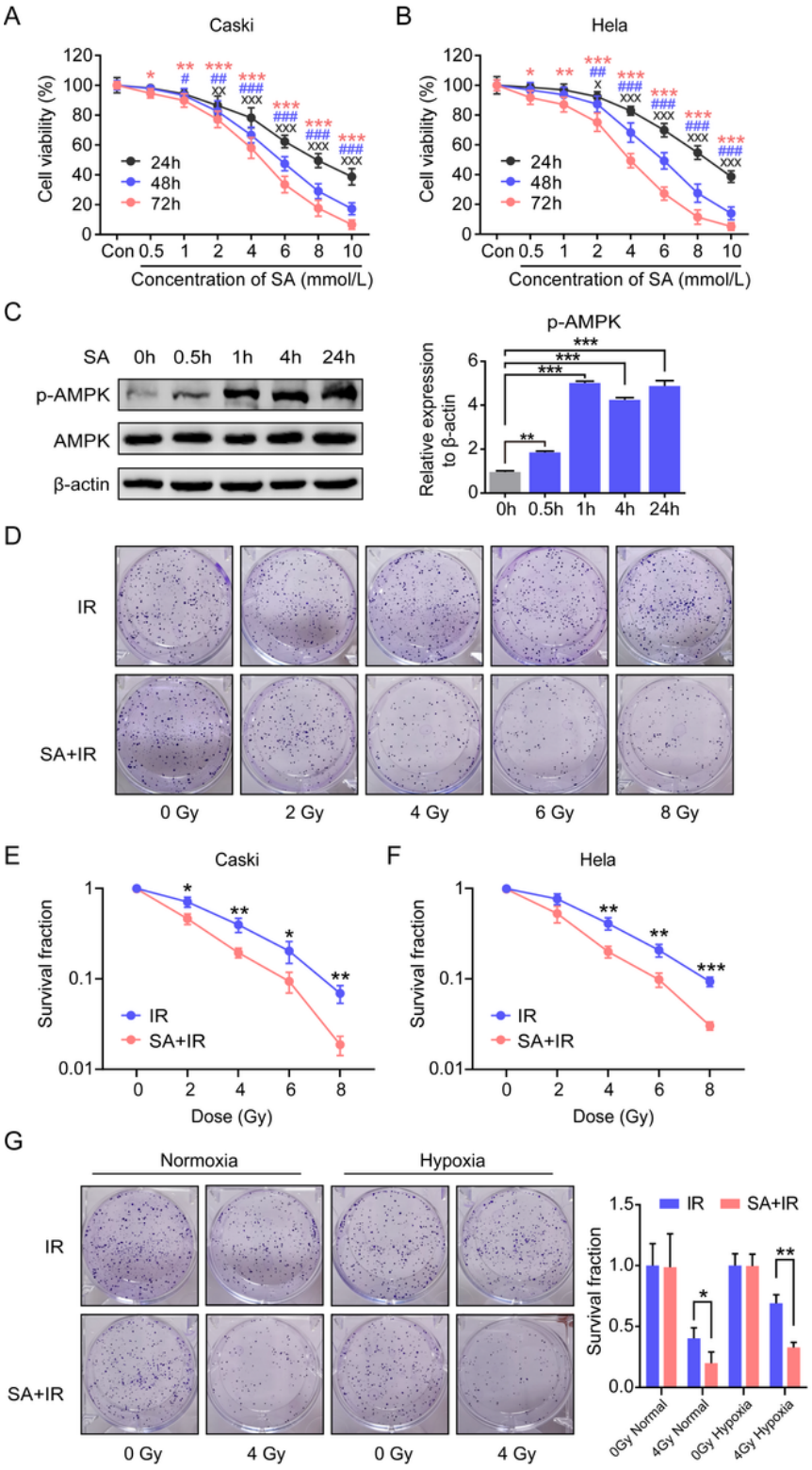

Figure 1

SA affects cervical cancer proliferation and response to radiation therapy (A and B) Viability rate of CaSki (A) or Hela (B) cells treated with SA at indicated concentrations for various time points. Statistically significant differences compared with corresponding control cells (not treated with SA) are shown (*P < 0.05, **P < 0.01, ***P < 0.001 for 24 h treatment group; #P < 0.05, ##P < 0.01, ###P < 0.001 for 48 h treatment group; xxP < 0.01, xxxP < 0.001 for 72 h treatment group respectively). (C) Time course of SA

effects on the level of AMPK phosphorylation. CaSki cells were treated with 4mM SA for 0.5-24h, the expression of p-AMPK and AMPK were examined by Western blot analysis. β-actin was used as a loading control. (D) Representative images of colony formation in CaSki cells treated with or without SA (4mM 1h before irradiation at indicated doses). (E and F) Clonogenic survival curves were generated for CaSki (E) or Hela (F) cells treated with SA for 1 h and then exposed to 2, 4, 6 or 8 Gy X-ray irradiation.(G) Cells from SA+IR group were pretreat with SA for 1h before irradiation, and hypoxic group were pretreat with CoCl2 (100umol/L) for 4h before exposing to 0, or 4 Gy irradiation.Representative images of colony formation for each tumor cell group are shown and the histograms indicate the SF of Caski cells exposed to radiation under normoxic and hypoxic conditions. Comparison of the inhibitory effects on colony formation by radiation under normoxia.Survival data was normalized to the unirradiated control cells. Throughout, data are expressed as the mean ± SD and are representative of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

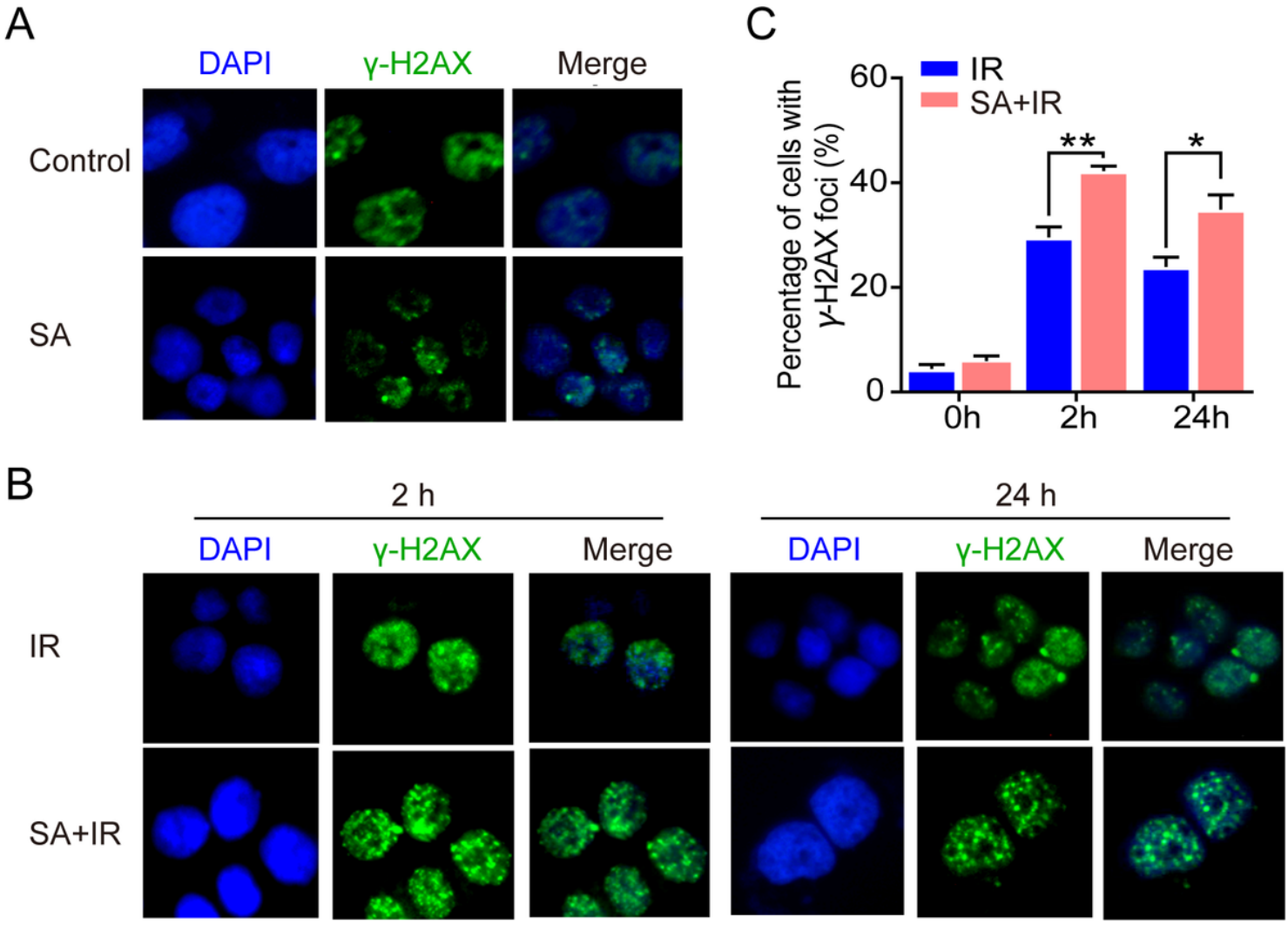

Figure 2

SA promotes IR-induced DNA double-strand breaks (A) Representative immunofluorescence images of nuclear γ-H2AX foci (cell nuclei: blue; γH2AX foci: green) in CaSki cells treated with or without SA. (B) Representative immunofluorescence images of different treatment-induced γ-H2AX foci (cell nuclei: blue;

γH2AX foci: green) in CaSki cells at 2 and 24 h after exposure to 8 Gy of irradiation. (C) Quantification of the number of γ-H2AX foci-positive cells after indicated treatments. Data represent the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01.

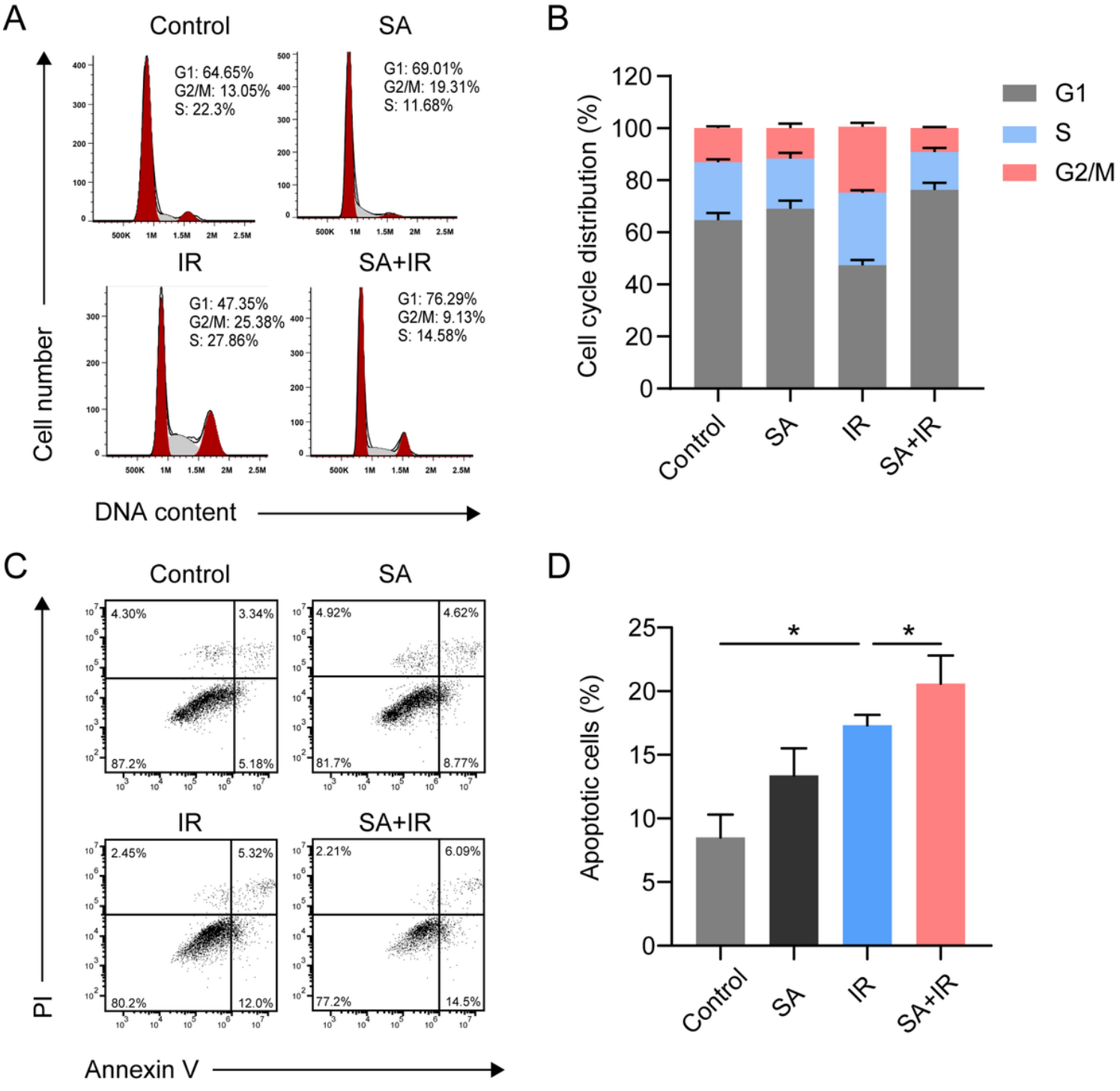

Figure 3

SA effects IR-mediated cell cycle and apoptosis (A) Cell cycle analysis was determined by BD Biosciences FACS Calibur Flow Cytometry in CaSki cells following different treatments. (B) Histogram plots show the percentage of cells distributing. (C) The apoptotic ratio of cells were detect at 48 hours after 4mmol/L salicylic acid and radiation treatment. The upper right and lower right quadrants represent proportion of

early and late apoptotic cells, respectively, which is added up to the total proportion of apoptotic cells. Numbers in plots indicate the percentage of cells for the indicated markers. (D) The apoptotic rate is represented as the percentage of Annexin V-positive cells.Data is presented as the mean ± SD of at least three independent experiments. *p < 0.05.

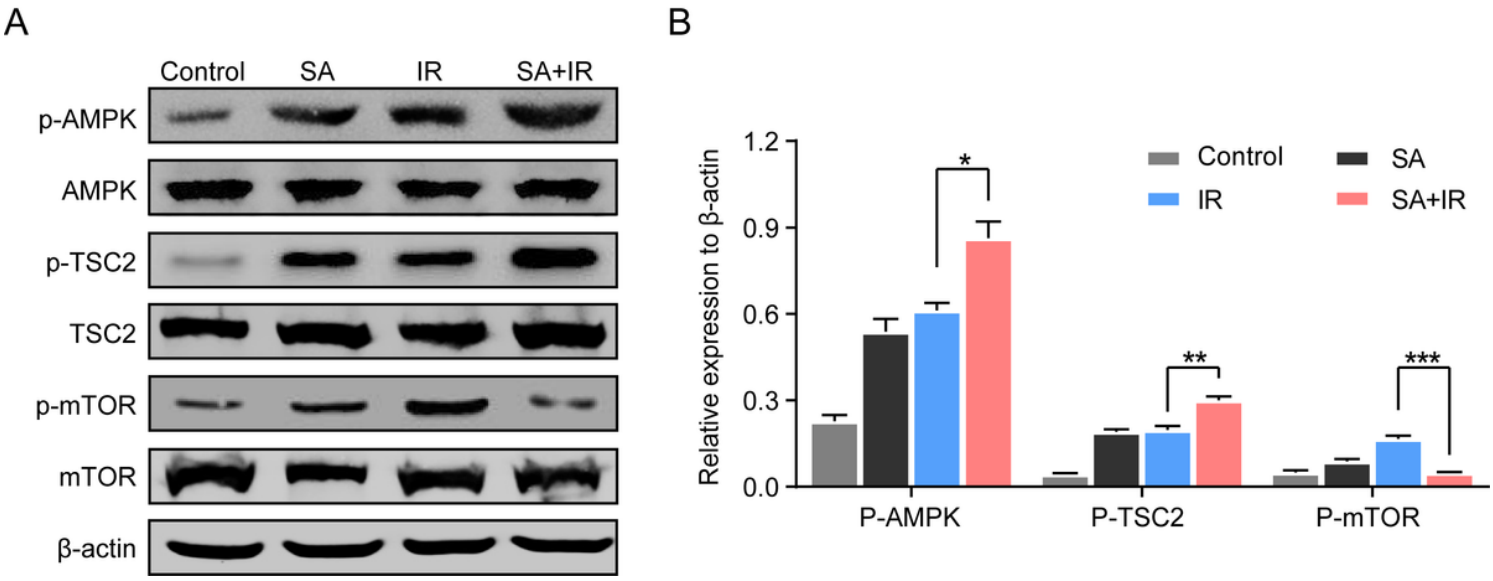

Figure 4

SA enhances the radiosensitivity of cervical cancer cell by activating AMPK/TSC2/mTOR pathway (A) The expression levels of p-AMPK, AMPK, p-TSC2, TSC2, p-mTOR, mTOR and β-actin proteins in CaSki cells were detected by Western blot. β-actin was used as a loading control. (B) The relative protein expression levels in A by grayscale analysis. Data represent the mean ± SD of three independent experiments. *p < 0.5, **p < 0.05, ***p < 0.005.