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AKBA inhibits radiotherapy resistance in lung cancer by inhibiting maspin methylation and regulating the AKT/FOXO1/p21 axis

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ABSTRACT

Acetyl-keto-b-boswellic acid (AKBA) functions in combating human malignant tumors, including lung cancer. However, the function of AKBA in regulating the radioresistance of lung cancer and its underlying mechanism still need to be elucidated. Radiation-resistant lung cancer cells (RA549) were established. Quantitative real-time polymerase chain reaction (QRT-PCR) and Western blot were employed to examine the messenger RNA (mRNA) and protein expressions. After being treated with AKBA and different doses of X-ray, cell proliferation and survival were examined using colony formation assay and cell-counting kit-8 (CCK-8) assay. The cellular localization of Forkhead box 1 (FOXO1) was measured by immunofluorescence (IF). Flow cytometry was employed to analyze cell cycle and apoptosis. In addition, *in vivo* experiment was performed to determine the effect of AKBA on the sensitivity of tumors to radiation. Herein, we found that AKBA could enhance the radiosensitivity in RA549, suppress cell proliferation, induce cell apoptosis and arrest cell cycle. It was observed that maspin was lowly expressed and hypermethylated in RA549 cells compared to that in A549 cells, while these changes were all eliminated by AKBA treatment. Maspin knockdown could reverse the regulatory effects of AKBA on radioresistance and cellular behaviors of RA549 cells. In addition, we found that AKBA treatment could repress the phosphorylation of Serine/Threonine Kinase (AKT), and FOXO1, increase the translocation of FOXO1 and p21 level in RA549 cells, which was abolished by maspin knockdown. Moreover, results of tumor xenograft displayed that AKBA could enhance the sensitivity of tumor to radiation through the maspin/AKT/FOXO1/p21 axis. We discovered that AKBA enhanced the radiosensitivity of radiation-resistant lung cancer cells by regulating maspin-mediated AKT/FOXO1/p21 axis.

Keywords: acetyl-keto-b-boswellic acid (AKBA); radioresistance; lung cancer; maspin; AKT/FOXO1/p21 axis

INTRODUCTION

Lung cancer is the leading cause of cancer death in China, and its incidence and mortality have increased significantly in recent years. It has been reported that approximately 2.1 million new lung cancer cases are diagnosed worldwide annually, and 1.8 million people die of lung cancer [1]. Radiotherapy is a widely used clinical treatment of lung cancer [2]. However, the results of many clinical treatments effects were poor because of the existence of radiotherapy resistance, which led to treatment failure, tumor recurrence, and distant metastasis [3]. It is suggested that enhancing the sensitivity of lung cancer cells to radiation acts as a key part in the clinical setting of treatment of lung cancer [4].

The Xihuang pill is a complementary and alternative medicine that has been used for the treatment of human malignancies since the 18th century. The Xihuang pill is composed of olibanum, commiphora myrrha, moschus, and calculus bovis. Acetyl-keto-b-boswellic acid (AKBA) has been identified as the most active compound in Xihuang pill [5]. As widely reported, AKBA has anti-tumor effects [6–8]. It was reported that AKBA could remarkably arrest the lung cancer cell cycle, induce cell apoptosis, and inhibit cell autophagy [7]. This suggested that AKBA has excellent anti-tumor effects on lung cancer, while the biological roles and potential mechanisms of AKBA in regulating the radiosensitivity of lung cancer remain unclear.

As is well known, DNA methylation is closely related to lung cancer progression and radiotherapy sensitivity. For example, it has been reported that the hypermethylation of NID2 is related to lung cancer cell malignant behaviors [9]. Additionally, hTERT promoter methylation promoted lung cancer development and radiotherapy resistance [10]. More importantly, it was previously reported that the anti-tumor effect of AKBA might in part be due to its ability to regulate methylation modifications of tumor-associated genes [11]. However, whether AKBA regulates the radiosensitivity of lung cancer by the regulation of DNA methylation modification is unclear.

Maspin, also called SERPINB5, belongs to serpin protease inhibitor family and functions in many biological processes [12, 13]. As widely reported, maspin functions as a tumor suppressor gene in many types of human malignancies [14]. In addition, it has been reported that maspin was related to radiosensitivity of cancer, specifically nuclear maspin expression in laryngeal carcinoma patients after postoperative radiotherapy was inversely proportional to the recurrence rate [15]. Notably, it was found that maspin existed in the form of hypermethylation modification in radioresistant lung cancer cells, and maspin knockdown elevated the resistance of radioresistant lung cancer cells to radiotherapy [16], revealing that the methylation modification of maspin is related to the radiotherapy resistance of lung cancer.

Serine/Threonine Kinase (AKT) is an oncogenic protein that regulates a range of cellular processes in cancer [17]. Many human malignancies, including lung cancer, have been found to have abnormally overexpressed or activated AKT, which is linked to increased cell proliferation [18]. It was reported that AKT knockdown inhibited lung cancer cell malignant behaviors as well as reduced p-Forkhead box 1 (FOXO1) expression [19]. FOXO1, as a downstream effector of insulin growth factor signaling, was inhibited by AKT activation [20]. In some kinds of cancer, FOXO1 arrests the cell cycle via increasing the cyclin-dependent kinase inhibitor p21 expression [21]. More notably, it was discovered that AKBA improved prostate cancer chemotherapy sensitivity by blocking AKT activation [8]. Furthermore, through suppressing the PI3K/AKT signaling pathway, AKBA displayed anticancer effects in lung cancer through cell cycle arrest, apoptosis induction, and autophagy suppression [7]. However, the roles of the AKT/-FOXO1/p21 axis in lung cancer radiosensitivity regulation, as well as the regulatory link between AKBA and the AKT/FOXO1/p21 axis in lung cancer, remains unknown.

Based on the above research, we speculated that AKBA could enhance the sensitivity of lung cancer cells to radiotherapy via repressing the methylation of maspin and regulating the AKT/FOXO1/p21 axis. Our work provided a reliable substance for lung cancer treatment and clarified its treatment mechanism.

MATERIALS AND METHODS

Cell culture and treatment

A549 cells were purchased from ATCC (VA, USA). Cells were cultured in DMEM (Gibco, MD, USA) containing 10% FBS (Gibco, MD, USA) at 37°C with 5% CO_2. A549 cells were irradiated every 2 weeks with 0, 2, 4 and 6 Gy, and each dose was repeated three times. Purified AKBA was purchased from the Duma Biotechnology (Shanghai, China), dissolved in DMSO (Sigma-Aldrich, MO, USA) at 20 mg/mL as a stock solution stored at −20°C until use. Musk ketone, bile acids, were obtained from Sigma (Sigma-Aldrich, MO, USA). 3-OAβBA purchased from Cayman Chemical (MI, USA). Alismol purchased from Macklin Biochemical (Shanghai, China). For AKBA treatment, cells were subjected to 10 μg/mL AKBA for 24 h. For musk ketone treatment, cells were subjected to 0.5 mg/mL for 24 h. For 3-OAβBA treatment, cells were subjected to 30 μg/mL for 24 h. For alismol treatment, cells were subjected to 10 μg/mL for 24 h. For bile acids treatment, cells were subjected to 50 μM bile acids for 24 h.

Establishment of radiation-resistant cells

A549 cells in the logarithmic phase were irradiated every 2 weeks with 0, 2, 4 and 6 Gy X-rays (Philips RT250 X-ray generator). After irradiation, the culture media was replaced, and after 5–7 days of traditional culture and passage, the cells' growth in each irradiation dose was observed. The survival cells were cultivated into first-passage subline cell. After receiving 10 or more sublethal doses of irradiation (total 56 Gy), the radiation-resistant cell line of A549 cells (RA549) were obtained.

Cell transfection

The short hairpin ribonucleic acid (RNA) of maspin (sh-maspin) and its negative control were transfected into cells with Lipofectamine™ 3000 (Invitrogen, CA, USA). The above plasmids were all obtained from GenePharma (Shanghai, China). At 24 h after transfection, quantitative real-time polymerase chain reaction (qRT-PCR) was employed to examine the transfection efficiency of plasmids.

Reverse transcription-PCR, subcloning, and sequencing

Total RNA of all cell lines was prepared with the RNeasy MIDI kit (Qiagen GmbH, Hilden, Germany) according to the instructions of the manufacturer. Reverse transcription was done in the presence of p53-specific primer (5′-CTCCCCACAACAAAACACCAG-3′) to generate cDNA covering the whole open reading frame of p53. Then the cDNA was amplified with p53-specific primers to recover the entire coding region of p53 (F: 5′-GGGAGCGTGCTTTCCACGACGG-3′, R: 5′-CCGGTCTCTCCCAGGACAGG-3′ and F: 5′-GCGTGTGGAGTATTTGGATGAC-3′, R: 5′- AGTGGGGAACAAGAAGTGGAG-3′). PCR products were gel purified and subcloned into the pBluescript KS II+ vector (Stratagene, La Jolla, CA). Positive clones (at least 10 clones from each clone) were used for sanger sequencing (ABI 377, Applied Biosystems, MA, USA).

Colony formation assay

Cells (1×10^3/well) were seeded on the six-well plates and incubated for two weeks. Colonies were fixed and stained with 25% (v/v) methanol and 0.1% (w/v) crystal violet for 10 min (Sigma-Aldrich), and the colonies formed were counted manually. Surviving fraction = (# of colonies counted / # of cells seeded) $_{\text{test}}$ / (# of colonies counted / # of cells seeded) $_{\text{0 Gy.}}$

Cell counting kit-8 assay

Cells were cultured in 24-well plates (2×10^4 cells/well) for 24 h and incubated with 10 μL of CCK-8 solution (Sangon, Shanghai, China) at 37°C for 3 h. Absorbance was analyzed at 450 nm with a microplate spectrophotometer (Bioteke, Beijing, China).

Cell cycle assay

Cells (1.5×10^6) were incubated with 70% ethanol for 30 min at 37°C. Cells were stained with 50 μg/mL PI (Sigma-Aldrich). Cells were analyzed using flow cytometry (Becton, Dickinson and Company, NJ, USA).

Cell apoptosis assay

Cells (1×10^6) were re-suspended in 500 μL of 1× Annexin-binding buffer (Beyotime, Shanghai, China) and then incubated with 10 μL Annexin V-FITC and 5 μL PI stain for 10 min. Samples were immediately analyzed using flow cytometry (Becton,Dickinson and Company).

Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 10 min and then sealed with goat serum for 30 min, followed by incubated overnight with antibody against FOXO1 (Abcam, 1:100, ab52857). Then cells were incubated with the secondary antibody (Abcam, 2:100, ab150077) for 1 h. Finally, DAPI (Sangon, Shanghai, China) was employed to stain the nucleus, and cells were sealed with the sealing liquid containing antifluorescence quenching reagent. The images were collected with a fluorescence microscope (Olympus, Tokyo, Japan).

Quantitative real-time polymerase chain reaction

Total RNA was extracted with TRIzol (Beyotime, Shanghai, China). The mRNA is reverse transcribed into cDNA with the Reverse Transcription Kit (Toyobo, Tokyo, China). Then, SYBR (Thermo Fisher Scientific) was employed for the qRT-PCR assay. β-actin was used as the reference gene for mRNA. The data was analyzed with $2^{-\Delta\Delta ct}$ method. The primers used in the study were listed as follows (5′-3′):

Maspin (F): GGAGGCCACGTTCTGTAT.
Maspin (R): CCTGGCACCTCTATGGA.
β-actin (F): CTCAGATGGTGTCTGCCATAG.
β-actin (R): CTCAGATGGTGTCTGCCATAG.

Western blot

Total proteins were extracted using RIPA (Thermo Fisher Scientific). BCA kit (Beyotime, Shanghai, China) was used to quantify the concentration. Equal amounts of protein mixed with loading buffer were boiled and loaded onto 10% SDS-page gel. The gel was transferred into a PVDF membrane (Millipore, MA, USA). The membranes were subsequently incubated overnight with antibodies against AKT (Abcam, 1:500, ab8805), p-AKT (Abcam, 1:1000, ab38449), FOXO1 (Abcam, 1:1000, ab52857), p-FOXO1 (Abcam, 1:1000, ab131339), p21 (Abcam, 1:1000, ab109119), CDK1 (Abcam, 1:1000, ab133327), Bcl-2 (Abcam, 1:2000, ab194956) and β-actin antibody (Abcam, 1:5000, ab8226) at 4°C. After being washed with PBS-T, membranes were then incubated with the corresponding secondary antibody (Abcam, 1:5000, ab7090, ab6789) for 60 min. Protein bands were analyzed by an ECL detection kit (Beyotime, Shanghai, China). Pictures were taken by Gel imaging system (Bio-Rad, CA, USA), then Imag J analyzed.

Methylation specific PCR

Cells were treated with sodium bisulfite. Then genomic DNAs were extracted by a DNA extraction kit (Tiangen, Beijing, China) then modified with bisulfite. The methylation specific PCR (MSP) reaction system, with total volume of 20 μL, consisted of 2 μL of template, 200 nmol/L each primer, 1 × PCR buffer, 4 mmol/L $MgCl_2$, 200 mmol/L dNTP and 1 U of Hot Star Taq DNA polymerase (Takara, Tokyo, Japan). The PCR conditions were 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 51°C for 30 s, 72°C for 1 min, and with a final extension cycle of 72°C for 10 min. The products of MSP were analyzed by agarose gel electrophoresis containing Invitrogen SYBR Safe DNA and visualized by GEL imaging system (Bio-Rad). The specific primers used for MSP were listed as follows:

Maspin (M forward): 5′-ATTTTTATTTTATCGAATATTTTAT TTTTCGGT-3′.

Maspin (M reverse): 5′-TACATACGTACAAACATACGTAC GACAATCCTCTCG-3′.

Maspin (U forward): 5′-TATTTTTATTTTATTGAATATTTTAT TTTTTGGT-3′.

Maspin (U reverse): 5′-TACATAC ATACAAACATACATACAAC AATCCTCTCA-3′.

U5 (F): 5′-TATTTTTATTTTATTGAATATTTTATTTTTTGG T-3′.

U5 (R): 5′-TACATACATACAAACATACATACAACAATCCTC TCA-3′.

Tumor xenograft *in vivo*

A total of 12 female BALB/c nude mice were purchased from SJA Laboratory Animal Co., Ltd (Hunan, China). 0.2 mL of cell suspension containing 1×10^7 RA549 cells was injected into the back of each mouse. When the tumor volume reached 100–150 mm^3, the tumors were irradiated with 6 Gy. Meanwhile, mice were randomly divided into two groups ($n = 6$) and treated once daily for 14 days with AKBA (100 mg/kg body weight) prepared in 0.5% carboxymethylcellulose sodium or vehicle control. Tumor sizes were recorded every 2 or 3 days. The tumor volumes were calculated as follows: $V = LW^2/2$. The mice were euthanized after 28 d. The animal studies were approved by our hospital.

Statistical analysis

All the tests conducted in this work were repeated at least three times. SPSS 19.0 (IBM, Armonk, NY) software package was applied for statistical data analysis and the measurement data were expressed as means ± standard deviation (SD). The differences among two groups were analyzed by Student's t-tests. One-way analysis of variance (ANOVA) was employed to evaluate the differences among multiple groups. The *P* values less than 0.05 were considered significant.

RESULTS

AKBA made RA549 cells more sensitive to irradiation, repressed cell proliferation, arrested the cell cycle and promoted apoptosis

RA549 cells were successfully established, and the survival ratio of RA549 cells was significantly increased compared to those of control cells exposed to X-ray irradiation (Fig. 1A–B). Next, the five monomer components of Xihuang pill were used in RA549 to screen the most radiosensitive in lung cancer cells. As shown in Fig. 1C–D, among the five monomers, AKBA presented the best effect in sensitizing radioresistant lung cancer cells to irradiation. Therefore, we selected AKBA for follow-up research. It was observed that the cell proliferation of RA549 cells was remarkably reduced by AKBA treatment (Fig. 1E). In addition, the population of cells in the G0/G1 phase was markedly elevated after AKBA treatment (Fig. 1F). Moreover, AKBA remarkably promoted the apoptotic rate of RA549 cells (Fig. 1G). Radiation often induces p53 mutations, and we detected the expression of p53 and the effect of AKBA treatment on p53 expression in RA549. We found p53 was lowly expressed in RA549 and was correlated to the radiation-caused mutation at exon5 and 6, but AKBA had no influence on the p53 expression level in RA549 compared to control (Supplementary Material1 Supplementary Fig. S1A–C and Supplementary Material2 Supplementary Fig. S2A). In summary, AKBA had the potential to reverse the radioresistance of lung cancer cells and was not dependent on p53, and this was consistent with the previous study [6].

AKBA increased maspin expression by reducing maspin methylation in radioresistant lung cancer cells

Maspin is reported to be related to radiosensitivity of laryngeal carcinoma [15]. Herein, we discovered that maspin expression was significantly reduced in RA549 cells in comparison to that in A549 cells (Fig. 2A–B), revealing that maspin was related to the radiosensitivity of lung cancer. As previously reported, maspin was hypermethylated in radioresistant lung cancer cells [16]. The result of MSP showed that maspin was hypomethylated in A549 cells while it was hypermethylated in RA549 cells (Fig. 2C). Notably, we found that AKBA treatment resulted in increased maspin expression in RA549 cells (Fig. 2D–E), as well as a reduced methylation level of maspin (Fig. 2F). In addition, it was found that the methylation inhibitor 5-AZA-dC remarkably increased the mRNA level of maspin in RA549 cells (Fig. 2G). Collectively, our results revealed that AKBA could increase maspin expression in RA549 cells by reducing maspin methylation.

AKBA inhibited RA549 cell proliferation through upregulation of the maspin/AKT/FOXO1/p21 axis

To explore the physiological effect of AKBA on maspin-mediated cell functions, sh-NC or sh-maspin was transfected into AKBA-treated RA549 cells. First of all, the mRNA and protein level of maspin in AKBA-treated RA549 cells was significantly reduced by sh-maspin transfection (Fig. 3A–B). In RA549 cells, AKBA treatment reduced p-AKT, p-FOXO1 and CDK1 levels but increased the level of p21, whereas maspin knockdown abrogated all of these effects (Fig. 3C). Meanwhile, p21 induction by AKBA is AKT-dependent rather than a p53-dependent response (Supplementary Material2 Supplementary Fig. S2B–C). As reported, FOXO1 presented in the cytoplasm constitutively in A549 cells, while AKT inhibition resulted in FOXO1 translocation to the nucleus and initiation of apoptosis [22]. In the current study, we found that AKBA could promote FOXO1 translocation to the nucleus in RA549 cells, which was abolished by maspin silencing (Fig. 3D). In addition, the inhibitory effect of AKBA on RA549 cell proliferation was abolished by maspin knockdown (Fig. 3E). Meanwhile, maspin silencing was found to remove the arresting impact of AKBA therapy on the cell cycle of RA549 cells (Fig. 3F). Furthermore, maspin knockdown eliminated AKBA induced cell apoptosis in RA549 cells (Fig. 3G). In conclusion, AKBA regulated cellular behaviors of RA549 cells by regulation of the maspin/AKT/FOXO1/p21 axis.

Maspin knockdown reversed the regulatory effects of AKBA on radioresistance of RA549 cells

As revealed in Fig. 4A–B, maspin knockdown abrogated the effects of AKBA treatment on the sensitivity of RA549 cells to radiotherapy. In order to detect the effects of AKBA combined with X-irradiation mediated radio-sensitization effects, 6 Gy was chosen for the function analysis. As shown in Fig. 4C, AKBA induced p21 expression, but inhibited CDK1 and Bcl-2 expression compared to X-ray alone, whereas maspin silencing terminated these effects. Meanwhile, maspin knockdown eliminated AKBA's apoptosis-promoting activity in RA549 cells under 6 Gy treatment (Fig. 4D). Taken together, AKBA sensitized RA549 cells to irradiation and promoted cell apoptosis by elevating maspin expression.

AKBA enhanced the sensitivity of xenograft tumor to radiation *in vivo*

Tumor xenografts in nude mice revealed that RA549 caused the tumor formation and the tumor volume reached 100–150 mm^3 on the day 10 (Fig. 5A and 5C). As displayed in Fig. 5B–D, after 6 Gy of X-ray radiation, tumor growth and weight were suppressed in the AKBA group compared to the control group. In addition, it was observed that AKBA resulted in an increased mRNA level of maspin in tumor tissues of mice exposed to irradiation (Fig. 5E). Furthermore, we found that AKBA led to the reduced p-AKT, p-FOXO1, and CDK1 levels and an increased p21 level in tumor tissues of mice exposed to irradiation (Fig. 5F). In total, AKBA could enhance the sensitivity of xenograft tumors to radiation via regulating the maspin/AKT/FOXO1/p21 axis.

DISCUSSION

Radiotherapy is one of the main treatment methods for advanced lung cancer [23]. Unfortunately, a variety of factors result in the radiation resistance of cancer cells, leading to increased radiation dose in order to maintain efficacy [24]. However, the increase in radiation dose also increases the risk of a series of radiotherapy-related complications [25]. Therefore, novel therapeutic approaches that decrease the radioresistance of lung cancer are urgently needed. As widely reported, AKBA inhibits the development of various human malignancies [26, 27]. Herein, the role of AKBA in reversing the radioresistance of lung cancer was studied. It was revealed for the first time that AKBA enhanced the sensitivity of lung cancer cells

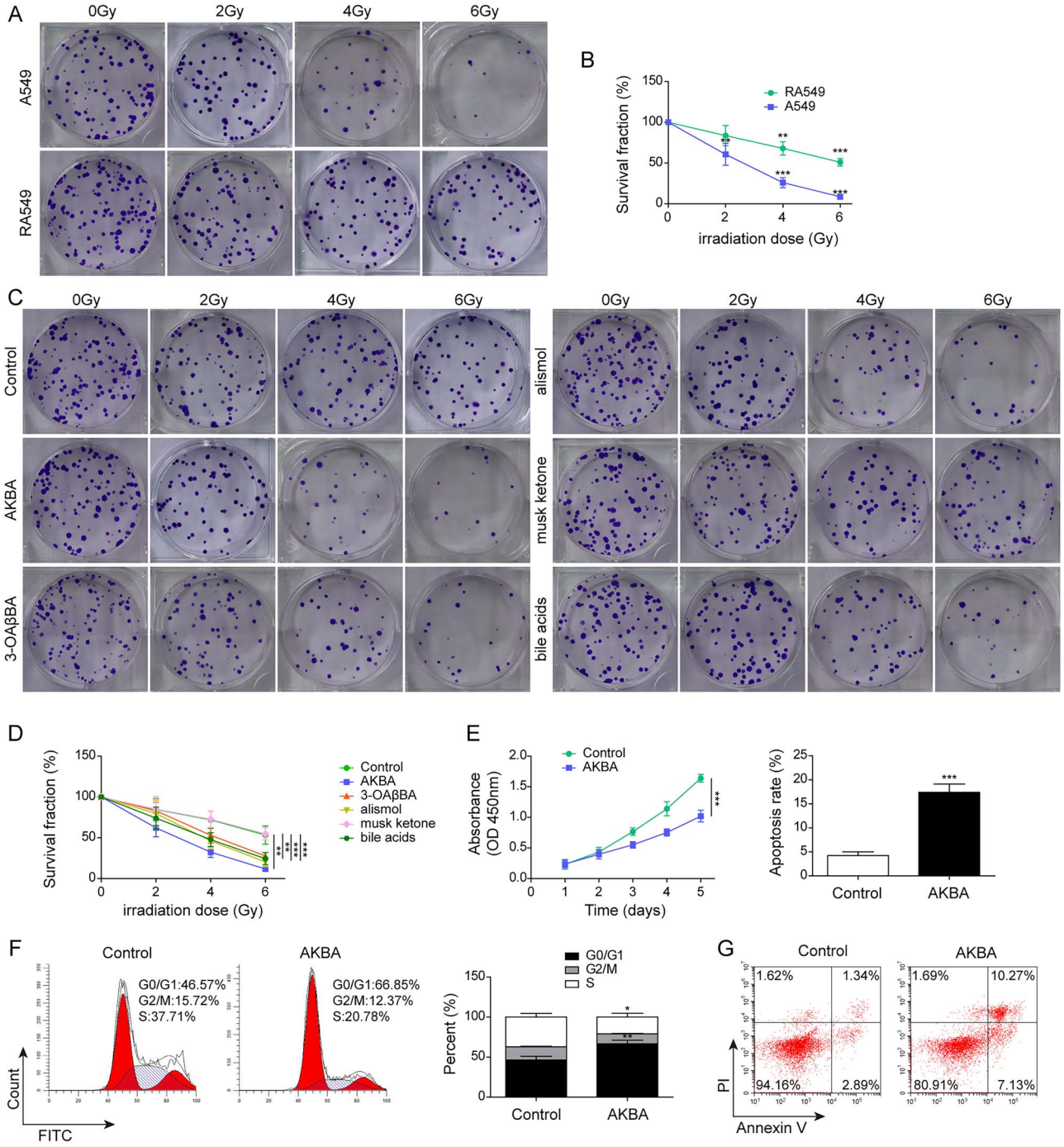

Fig. 1. AKBA made RA549 cells more sensitive to irradiation, repressed cell proliferation, arrested the cell cycle and promoted apoptosis. (A-B) Cell survival fraction of RA549 and A549 cell treated with radiation were analyzed using colony formation assay. (C-D) Cell survival fraction of RA549 cells were then measured by colony formation assay after the cells were treated with AKBA, 3-OaβBA, alismol, musk ketone or bile acids and then exposed to irradiation. (E) CCK-8 assay was carried out to examine the proliferation of RA549 cells after AKBA treatment. (F) Cell cycle was analyzed by flow cytometry. (G) Cell apoptosis was determined by flow cytometry. The measurement data were presented as mean ± SD. All data was obtained from at least three replicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

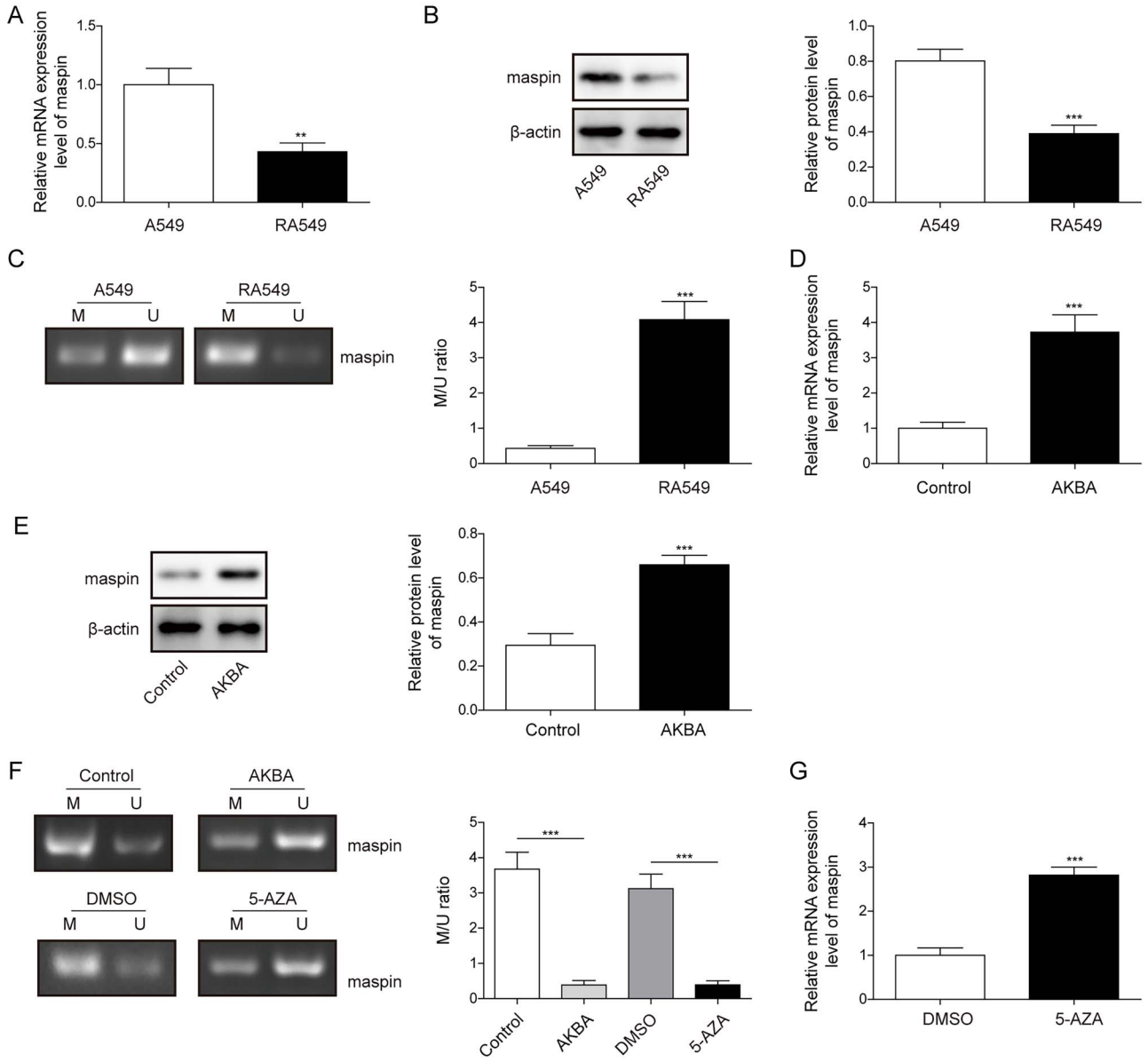

Fig. 2. AKBA increased maspin expression by reducing maspin methylation in radioresistant lung cancer cells. (B) QRT-PCR and Western blot were employed to detect maspin expression in A549 and RA549 cells. (C) The methylation level of maspin in A549 and RA549 cells was analyzed by MSP and the M/U ratio was analyzed by ImageJ (M represented methylated DNA and U represented unmethylated DNA). (D-E) Maspin expression in RA549 cells following AKBA treatment were analyzed by qRT-PCR and Western blot. (F) The methylation level of maspin in RA549 cells following 5-AZA-dC treatment or AKBA treatment were analyzed by MSP. (G) QRT-PCR was performed to detect maspin expression in RA549 cells after 5-AZA-dC treatment. The measurement data were presented as mean ± SD. All data was obtained from at least three replicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

to radiotherapy by repressing maspin methylation and regulating the AKT/FOXO1/p21 axis.

As a well-known traditional Chinese medicine formula, Xihuang pill is widely used for tumor treatment in China [28]. The Xihuang pill is composed of olibanum, commiphora myrrha, moschus, and calculus bovis [8]. In the present study, we found that among the five monomers of Xihuang pill, AKBA had the best effect in sensitizing radioresistant lung cancer cells to irradiation. AKBA, as a terpenoid isolated from natural plants, is widely used in the treatment of various inflammatory diseases [29, 30]. Recently, it has been widely reported that AKBA has the ability to promote cell apoptosis of cancer cells, such as prostate cancer [8], colon cancer [6], and glioblastoma

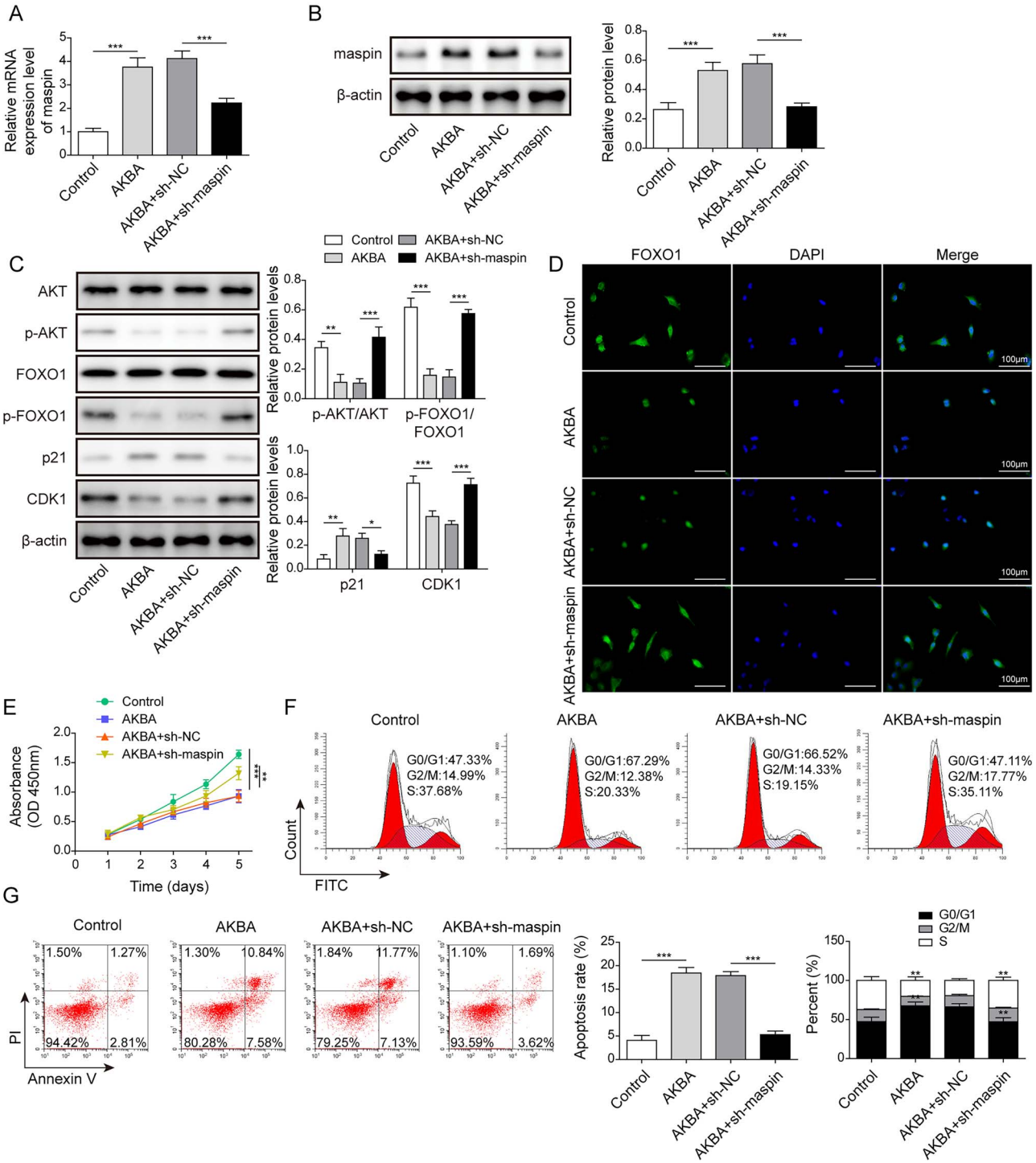

Fig. 3. AKBA inhibited RA549 cell proliferation through upregulation of the maspin/AKT/FOXO1/p21 axis. AKBA-treated RA549 cells were transfected with sh-NC or sh-maspin. (A-B) Maspin expression was examined by qRT-PCR and Western blot. (C) AKT, p-AKT, FOXO1, p-FOXO1, p21 and CDK1 levels were assessed by Western blot. (D) The location of FOXO1 was analyzed by IF. (E) CCK-8 assay was employed to determine the proliferation. (F-G) Flow cytometry was conducted to detect cell cycle and cell apoptosis. The measurement data were presented as mean ± SD. All data was obtained from at least three replicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

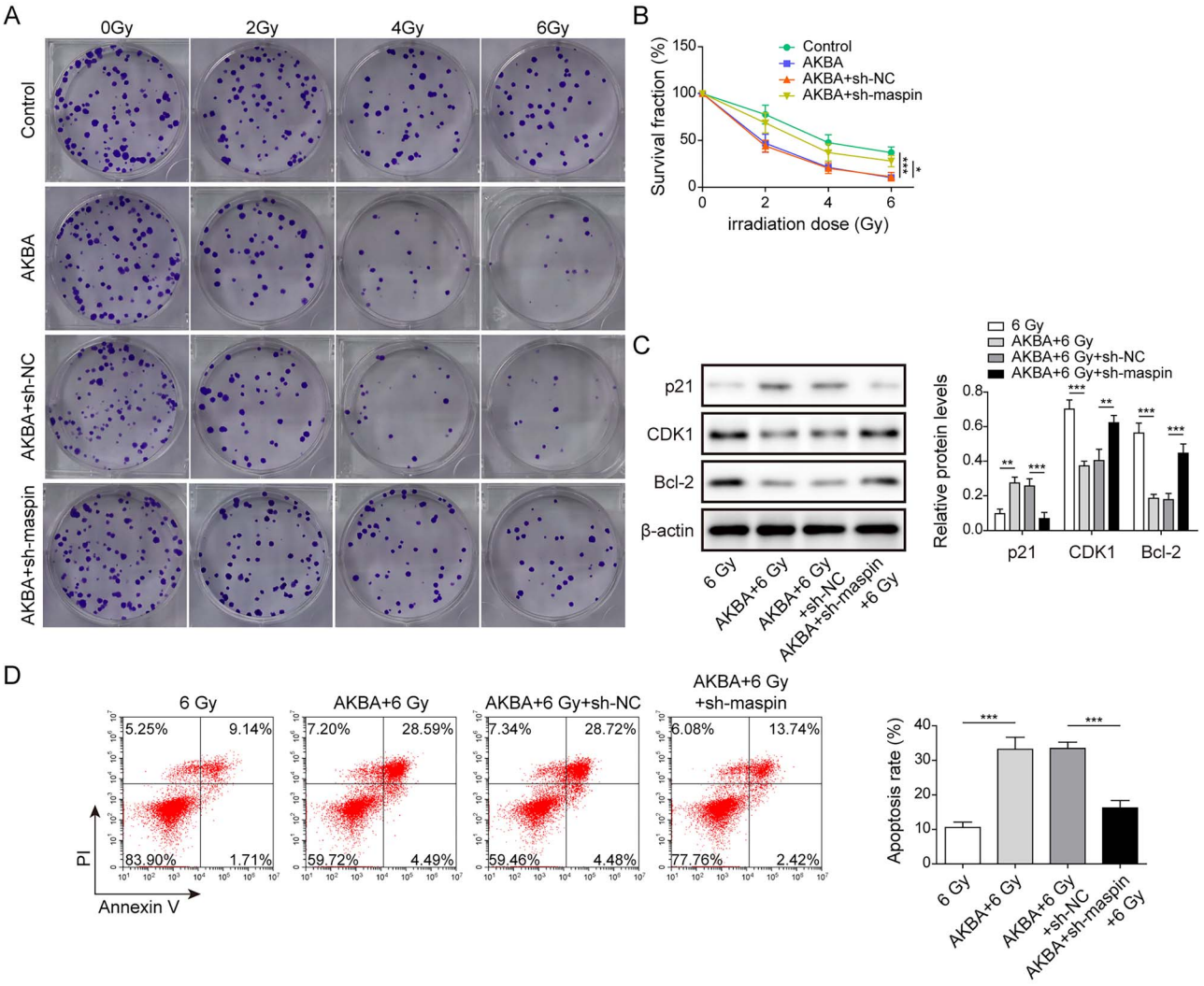

Fig. 4. Maspin knockdown reversed the regulatory effects of AKBA on radioresistance of RA549 cells. Sh-NC or sh-maspin was transfected into AKBA-treated RA549 cells. (A-B) Cell survival fraction was determined by colony formation assay. (C) Western blot detected the expression of p21, CDK1 and Bcl-2 in RA549 under 6 Gy treatment. (D) Cell apoptosis was determined by flow cytometry. The measurement data were presented as mean ± SD. All data was obtained from at least three replicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

[28]. In addition, previous studies revealed that AKBA showed anti-cancer effects by arresting the cell cycle [31, 32]. Notably, it was reported that AKBA could inhibit lung cancer progression by arresting the cancer cell cycle, promoting cell apoptosis and repressing cell autophagy [7]. All these studies revealed that AKBA functioned in combating diverse human cancers, including lung cancer. In the current study, it was found that AKBA could increase the radiosensitivity of tumor cells. In addition, our results demonstrated that AKBA could reduce cell proliferation, promote cell apoptosis, and arrest the cell cycle of radioresistant lung cancer cells. As a natural ingredient, AKBA has the advantages of low toxicity, low side effects, and low cost, and has good clinical application prospects. Therefore, it is worth studying whether it is capable of improving the efficacy of clinical treatment of advanced lung cancer when combined with radiotherapy.

SERPINB5, also called maspin, has multiple functions as a tumor suppressor. It has been reported that maspin is capable of suppressing cancer cell proliferation, reducing tumor angiogenesis and increasing tumor sensitivity to drug-induced apoptosis in lung cancer [33]. In addition, it was previously reported that maspin upregulation could arrest the cell cycle of cancer cells [34]. Moreover, it was observed that maspin expression was directly correlated with chemoradiotherapy response in head and neck carcinoma [35]. However, the mechanism and role of maspin in AKBA-meditated anti-radioresistance activity for lung cancer have yet to be elucidated. Herein, we observed that maspin was significantly downregulated in RA549 cells in comparison to that

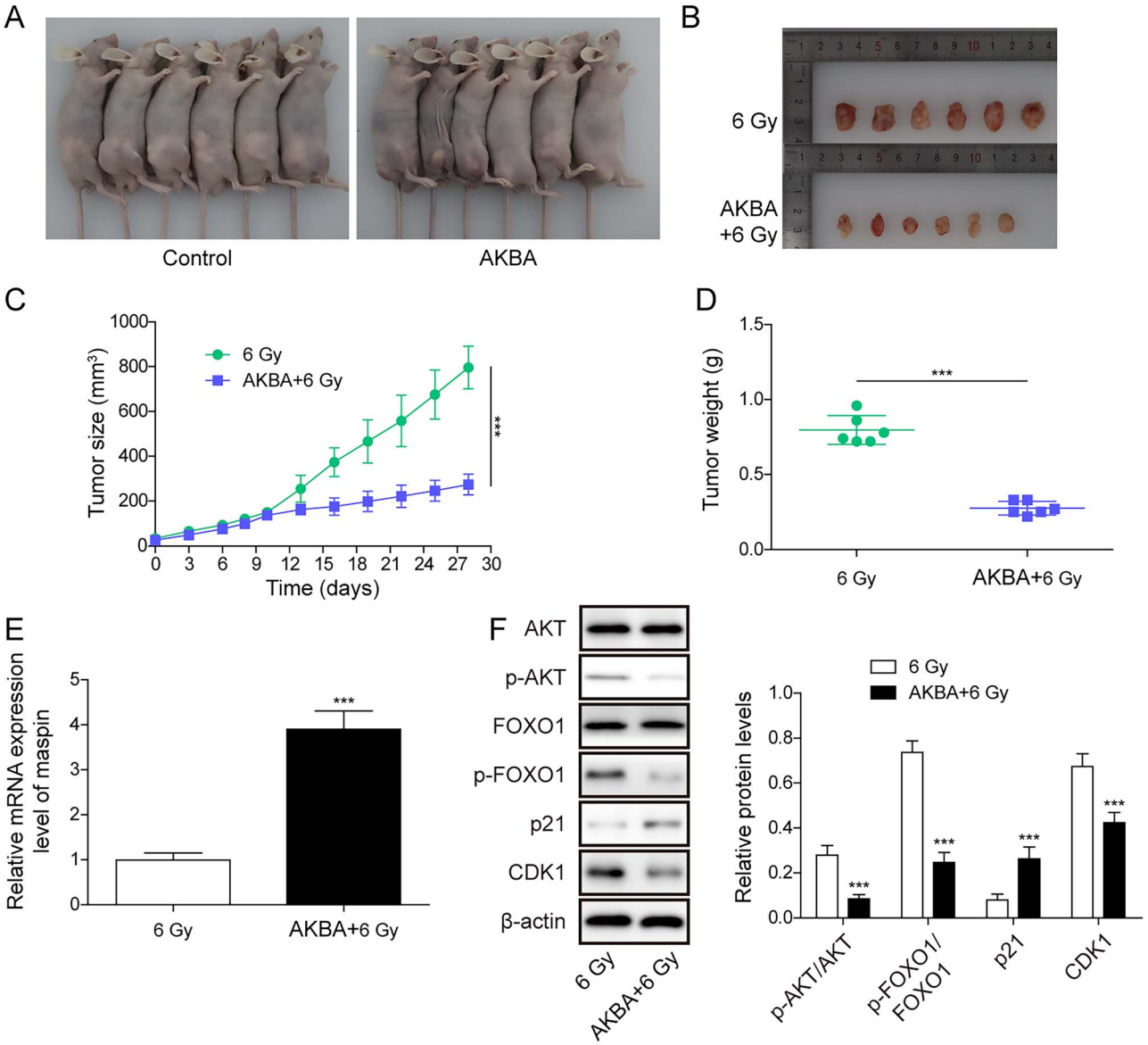

Fig. 5. AKBA enhanced the sensitivity of xenograft tumor to radiation *in vivo*. RA549 cells were inoculated into nude mice, the tumors were irradiated with 6 Gy, meanwhile mice were treated with AKBA. (A, C) Real-time monitoring of the transplanted tumor growth. (B, D) The tumors were collected, and the size and weight of tumors were measured. (E) Maspin expression was assessed using qRT-PCR. (F) Western blot was performed to examine AKT, p-AKT, FOXO1, p-FOXO1, p21 and CDK1 levels. The measurement data were presented as mean ± SD. $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

in A549 cells, suggesting that maspin was related to the radiosensitivity of lung cancer. Previously research reported that maspin existed in the form of hypermethylation modification in radioresistant lung cancer cells [16]. Furthermore, the anti-cancer effects of AKBA have been linked to its ability to regulate methylation modifications of tumor-associated genes [11]. Interestingly, our results revealed that maspin methylation levels were remarkably elevated in RA549 cells in comparison to that in A549 cells. Moreover, it was observed that AKBA treatment resulted in increased maspin expression in RA549 cells as well as a reduced methylation level of maspin. Then, loss-function experiments revealed that maspin silencing reversed the regulatory effects of AKBA on radioresistance and cellular behaviors of RA549 cells. Our study provided evidence that AKBA promotes maspin expression by reducing maspin methylation levels, thereby enhancing the sensitivity of lung cancer cells to radiotherapy.

It has been widely reported that the activation of AKT pathway results in various hallmarks of lung cancer, such as cancer cell apoptosis suppression, sustained angiogenesis, enhanced cell invasion and metastasis and insensitivity to antigrowth signals [36]. It was also reported that AKT pathway activation was related to the

radioresistance of cancers [37, 38]. AKT signal transduction occurs through downstream effectors such as FOXO1 [18]. In addition, Shao *et al.* revealed that the repression of AKT and FOXO1 phosphorylation could increase both the prototype and nuclear translocation of FOXO1 could promote the apoptosis of human glioma cells [39]. As well known, FOXO1 is a cell-cycle regulator [40]. P21, as a cyclin-dependent kinase inhibitor, is involved in arresting the lung cancer cell cycle, suppressing cell proliferation and promoting cell apoptosis [41]. As previously reported, FOXO1 could induce G1 cell cycle arrest by upregulating p21 in some types of cancer [21]. Therefore, it is suggested that the AKT/FOXO1/p21 axis may be involved in regulating the radioresistance of lung cancer. More importantly, previous studies demonstrated that AKBA presented anti-tumor effects on human malignancies, including lung cancer, at least in part by repressing the AKT pathway [7, 27, 42]. In the current study, it was observed that AKBA could suppress AKT and FOXO1 phosphorylation while increasing p21 expression and FOXO1 translocation into nucleus, but was independent of p53 in RA549 cells. This suggests that AKBA might regulate radioresistance and cellular behaviors of RA549 cells by affecting the AKT/FOXO1/p21 axis. In addition, our results demonstrated that the inhibitory effect of AKBA treatment on the phosphorylation of AKT/FOXO1 and CDK1 expression, as well as the promoting effect of AKBA on p21 expression, were all reversed by maspin silencing. It was concluded that AKBA regulated the AKT/FOXO1/p21 axis by acting on maspin, thereby regulating radioresistance and cellular behaviors of RA549 cells.

Taken together, our research reported for the first time that the anti-tumor effects of AKBA on reversing radioresistance of radioresistant lung cancer cells and repressing the malignant behaviors of cancer cells, the underlying mechanism of which was regulating maspin-mediated regulation of the AKT/FOXO1/p21 axis. Our findings provided support for the improvement of radiotherapy resistance in the clinical treatment of advanced lung cancer.

SUPPLEMENTARY DATA

Supplementary data is available at *RADRES Journal* online.

AVAILABILITY OF DATA AND MATERIAL

All data generated or analyzed during this study are included in this published article.

STATEMENT OF ETHICS

All animal experiments were undertaken in accordance with The First Affiliated Hospital of Hunan University of Chinese medicine.

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CONFLICT OF INTEREST

All authors agree with the presented findings, have contributed to the work, and declare no conflict of interest.

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