Saikosaponin-d Increases the Radiosensitivity of Hepatocellular Carcinoma Cell line SMMC-7721 by Adjusting the G0/G1 and G2/M Checkpoints of Cell Cycle

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Abstract:

**Background:** Saikosaponin-d (SSd) is a monomer terpenoid, purified from Chinese herbal drug Radix bupleuri, with multiple effects including anticancer. But the effect of SSd on the tumor exposed to radiation is largely unknown. To investigate the radiosensitive effect of SSd and its possible mechanism, we combined SSd with radiation therapy to treat hepatocarcinoma cell SMMC-7721 under oxia and hypoxia.

**Methods:** The cell growth, cell apoptosis and cell cycle distribution were examined after treatment with SSd or radiation alone and their combinations in oxia and hypoxia. The protein and mRNA levels of p53, Bcl2 and BAX were measured by Western blot and RT-PCR respectively.

**Results:** In either oxia or hypoxia, SSd and radiation alone exposure resulted in a cell growth inhibition and increased apoptosis rate at the concentration used. And when treated with the combination of SSd and radiation, these effects were enhanced. Moreover, SSd not only potentiated the effects of radiation to induce Go/G1 arrest in SMMC-7721 cells, but also reduced the G2/M-phase population in hypoxia. However, in oxia, SSd only potentiated the effects of radiation to induce Go/G1 arrest but not in G2/M-phase. In addition, these effects resulting from SSd or radiation and their combinations were accompanied with up-regulated expression of p53 and BAX, a down-regulation of Bcl2 in
either oxia or hypoxia.

**Conclusion:** It is demonstrated unambiguously that SSd potentiated the effect of radiation in this cell line and would be a promising radiosensitizer. Radiosensitizing effect of SSd may contribute to its function to affect Go/G1 and G2/M checkpoints of cell cycle.

**Keywords:** Hypoxia; HCC; Radiation; Cobalt chloride; HIF-1α

**Background**

Primary hepatocellular carcinoma (HCC), which comprises 90% of all malignant tumors developed in the liver is the most devastating of human malignancies that cause death within a few months unless treated properly [1,2]. Nevertheless, if it is diagnosed at the early stage and treated by surgical resection or transcatheter arterial chemo-embolization (TACE), the survival rate can be expected to improve [3]. However, there are considerable patients with HCC who are unsuitable or ineffective for surgery or TACE, and must be treated with some other kinds of treatment. Radiotherapy has long been used in cancer therapy and has been frequently applied for patients with HCC [4,5]. To achieve maximum biological response from the radiation therapy, oxic circumstance is important to cancer cells and tissues. However, hypoxia is a common feature of the solid human tumor, and causes resistance to radiation in cancer cells during radiation therapy.

To overcome the hypoxic resistance, several approaches have been developed to alter hypoxic status of cancer cell during radiation therapy over several decades. Currently, numerous new hypoxic radiosensitizers have been developed and some of them have been in clinical evaluation [6]. Also, many herbs and other botanical formulations have been constantly, at the same time, developed into radiosensitizer or hypoxic sensitizer [7]. Nevertheless, the clinical utility of the radiosensitizers is still disputable.

It has been reported that saikosaponin-d (SSd), an extraction from a type of traditional Chinese herbs, Bupleurum chinensis DC, exhibits a variety of pharmacological properties, such as anti-inflammation [8,9], preventing hepatocyte injury [10,11], inhibiting angiogenesis [12], and anti-cancer [13,14,15,16]. Our recent clinical practice of combining SSd administration with radiation in treating patients with hepatocellular carcinoma revealed that this joint treatment was more effective than either monotherapy alone, indicating a contributory effect of SSd on radiotherapy. However, the molecular mechanisms of SSd’s radio-sensitization effect on hepatocellular carcinoma cell remains unclear. To investigate the radiosensitive effect and therapeutic efficacy of SSd, we combined SSd with radiation therapy to treat hepatocellular carcinoma cell line SMMC-7721 under oxic and hypoxic conditions.
Methods

Cell Culture

Cells were cultured in RPMI-1640 medium (PAA Laboratories GmbH, Austria) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G and 100 µg/ml streptomycin sulfate (GIBCO, Invitrogen) in a humidified atmosphere containing 5% CO₂ at 37°C and passaged once per 2-3 d. During logarithm growth period the cells were collected by 0.25% trypsin digestion. After counting living cells, the cell density was adjusted to 5×10⁴/mL, and propagated by seeding them in 96-well plates for further treatment including normoxic and hypoxic incubation. Normoxic culture was performed by incubating the cells in RPMI-1640 medium with 10% FBS at 37°C. For induction of hypoxia, cells grown to 80-90% confluence were trypsinized and counted, and seeded into a 6-well plate (5×10³ cells/well) followed by an incubation with 100 µM Cobalt chloride (CoCl₂) for 4 h in 4 ml serum-free medium before X-ray irradiation at a dose rate of 400 cGy/min (Clinac 2100EX; Varian Medical Systems Inc., CA) [17,18].

SSd preparation and experimental groups

Saikosaponin-d (SSd) and CoCl₂ were obtained from Sigma Chemical (St. Louis, MO). SSd was dissolved in dimethylsulfoxide (DMSO, Sigma, St Louis, MO, USA) and stored at -20°C. Desired final concentrations were achieved by dilution with RPMI-1640. SMMC-7721 cell were treated with radiation, SSd, PX-478 (PX, Millipore, USA) alone or a combination of radiation and SSd or PX-478. Radiation was performed by 6 MV X-ray irradiation equipment (Clinac 2100EX; Varian Medical Systems Inc., CA) at a dose rate of 400 cGy/min. The distance between the cells and the radiation source was maintained at 0.1 meter. SSd was administered at different concentrations. For joint treatment, the dose of radiation was set to be 2 Gy, the regular clinical radiation dose inducing moderate cell apoptosis rate. SSd was added to the cultures 2 h prior to radiation. Control cultures received the carrier solvent consisting of 0.1% DMSO.

Cell Viability Assay

Cell viability was measured by MTT assay under oxic condition or CoCl₂-induced hypoxic condition after 4 h of chemical hypoxic culture. SMMC-7721 cell were seeded into a 96-well plate (5×10³ cells/well) and incubated at 37°C in 5% CO₂ for different periods of time as designed. Then, MTT solution (5 mg/ml) (Sigma, St Louis, MO, USA) was added (20µl/well) and the cells were incubated for another 4 h. Supernatants were removed and formazan crystals were dissolved in 200µL of DMSO. Finally, optical density was determined at 492 nm using multi-microplate test system (POLARstar OPTIMA, BMG Labtechnologies, Germany). Inhibition rate of cell growth was calculated and plotted using the average data in quadruplicate.

FCM Apoptosis Assay
Following treatment, cells were harvested and the pellet was suspended in 100 µL binding buffer, according to the manufacturer’s instructions of Annexin V-FITC/PI kit (BD Biosciences, USA), stained with 5µL Annexin V-FITC and 10µL PI, and then incubated for 15 min at room temperature. After another 400 µL binding buffer was added and the cells were transferred into BD Falcon® tube, the percentage of apoptotic cells in each sample was analyzed by FACSCaliburMT flow cytometer (Becton Dickinson Technologies, USA).

**FCM Cell Cycle Analysis**

Following treatment, cells were harvested, fixed with ice-cold 70% ethanol and stored at -20°C until the day of analysis. Cells were then washed in phosphate-buffered saline, and re-suspended in PI solution at 50 µg/mL containing 150 µg/mL RNase A for 15 min at 37°C, and analyzed by FCM using FACSCaliburMT flow cytometer (Becton Dickinson Technologies, USA) and ModFit program (Verity Software House, Topsham, MN).

**Western Blotting Analysis**

Following treatment, cell lysates were prepared with RIPA buffer containing protease inhibitor cocktail. Homogenates were centrifuged for 10 min at 20,000g and the supernatants were collected. Protein concentrations in the supernatants were determined by NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc.). Total cell lysates were subjected to SDS–PAGE on 10% SDS–acrylamide gel. Separated proteins were transferred to PVDF membranes (Millipore, USA) and incubated with primary antibodies against P53, bcl2 and Bax followed by incubation with an HRP-conjugated secondary antibody (Santa Cruz, CA). The membrane was stripped and incubated again with a primary antibody against β-actin (Santa Cruz, USA). Bands of Protein and β-actin were visualized with Thermo Scientific Pierce® Chemiluminescence Substrate and optic density (OD) value of the band were detected by CCD camera, recorded and quantified with Syngene G Box (Syngene, UK) and compared with relative control β-actin.

**Real Time PCR Detection**

Following treatment, total RNA was isolated from SMMC-7721 cells using the Trizol Reagent® (Invitrogen Life Technologies, USA) according to the manufacturer’s instruction. The quality and quantity of RNA sample were detected by NanoDrop® ND-1000 Spectrophotometer. The cDNA was synthesized using TaqMan® Reverse Transcription Reagents (ABI Life Technologies, USA) from 1 µg of RNA. RT –PCR detection was done on the ABI 7300 system using the SYBR® Green PCR Master Mix kit and predesigned primer/probe pairs for P53, bcl2 Bax and β-actin (Santa Cruz Biotechnology, Inc.). The sequences of primers were as follows: p53 (Forward: 5’-CCACCATCCACTACAACCTACAT-3’, Reverse: 5’-AGGACAGGCACACACAGCG-3’), bcl2 (Forward: 5’-CAATGCTGGACTGAAAAATTGTA-3’, Reverse: 5’TATT TTCTAAGGACGGCATGATCT-3’), BAX(Forward:5’-GACACCTGAGCTGAAGACT-3’, Reverse: 5’-GACACCTGAGCTGACCTTG-3’).
G-3', Reverse: 5'-GAGGAAGTCCAGTGTCCAGC-3'), and β-actin (Forward: 5'-TGGCACCCAGCAATGAA-3', Reverse: 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3').

The reaction mixture (25µL) contains 12.5µL SYBR Green PCR Master Mix, 1µL(10µM) each forward primer, 1µL(10µM) each reverse primer, 2µL 100ng each cDNA template and 8.5µL ddH2O. Thermal circulation parameters were 95° for 10 minutes for Taq polymerase activation followed by 40 cycles of 95° for 15 seconds, 60° for 1 minute. Normalization and analyses of fold change in target gene expression were carried out with β-actin using the internal reference by the 2^−∆∆CT method [19] and the Applied Biosystems GeneAmp 5700 SDS software.

**Statistical Analysis**

All values were expressed as the mean ± standard deviations (SD). Statistical analysis was performed with student’s t-test using the statistical software SPSS 13.0. P<0.05 was considered as statistically significant.

**Results**

**SSd Inhibited Cell Growth**

In the first experiment, the cell viability was determined with MTT assay. Either in oxia or hypoxia, SSd or radiation alone can inhibit the growth of SMMC-7721 cells, and compared with the control, the inhibitory effect was further enhanced with the combinations of SSd and radiation (Fig.1. p<0.01). We found that SSd in low concentrations of 1µg/ml showed no significant effects on the inhibition of hepatoma cells either in oxia or hypoxia, but, when combined with radiation, the inhibitory effect of hepatocellular carcinoma increased significantly, and its sensitivity to radiation elevated, especially under hypoxic condition, which is more significant than that under normoxic condition. Also, the effect was more significant at 3 µg/mL of SSd than that at 1 µg/mL. These results indicated that the combination treatment induced a significant cell growth inhibition, in a dose dependent manner. In oxia, exposure to radiation followed treatment with 1 µg/mL SSd, the inhibition rate increased from 17.9±3.42 % to 23.1±3.85%; in hypoxia, the inhibition rate increased from 12.8±3.01 % to 27.8±4.52%. These results indicated that the efficacies of combination treatment were more potent than the alone treatment, especially under hypoxia condition.

**SSd Promoted Cell Apoptosis**

The apoptosis of SMMC-7721 cell induced by SSd, radiation or their combination was determined by flow cytometry. PX-478, an inhibitor of HIF-1α, was used as a positive control. Treatment with SSd at the concentration of 3µg/ml significantly induced the apoptosis of SMMC-7721 in both oxia and hypoxia conditions, compared with control (Fig.
2.A, B p<0.05). Also, no significant differences were observed between oxia and hypoxia in the cell apoptosis induced by SSd alone (p>0.05), although cell apoptosis in oxia showed a more increased potential than that in hypoxia. The percentage of apoptotic cells induced by radiation at the dose of 2Gy was higher under oxia condition than that under hypoxic conditions (Fig. 2.A, B p<0.05). Critically, the combination therapy of SSd with radiation (2GY) demonstrated greater induction of apoptosis in SMMC-7721 cell under both oxia and hypoxia conditions, compared with either SSd or radiation therapy alone(Fig. 2.A, B p<0.01). Especially in hypoxic conditions, enhancing effect of radiation-induced apoptosis of hepatoma cells in the combined treatment was more pronounced compared to normoxic conditions (Fig. 2. A, B, P <0.05). In addition, we also found that under hypoxic condition, radiation-induced apoptosis of hepatoma cells was significantly enhanced after joining the PX-478, but this enhancement does not appear in normoxic conditions.

**SSd Altered Cell Cycle Distribution**

In order to investigate the underlying mechanism of the SSd to potentiate the apoptosis-inducing effect of radiation in SMMC-7721 cell, we analyzed the alteration in cell cycle using flow cytometry after every intervention. The results are shown in Figure 3. In oxia group, after the alone exposure of SSd or radiation, G0/G1-phase cells increased, but S phase population decreased (Fig. 3.A, C p<0.05). However, the percentage of G2/M-phase cells showed no significant alteration (p>0.05). These results suggest that G1-phase arrest resulted in the decrease in S-phase population. Moreover, when treated with the combination of SSd and radiation, the Go/G1 arrest further increased and S-phase population decreased(Fig. 3.A, C. p<0.01). In hypoxia, SSd or radiation alone and their combinations also induced G0/G1 arrest and S-phase population reduction, and treatment with the combined of SSd and radiation also further enhanced the cell cycle arrest than their exposure alone. In addition, after radiation alone exposure, G2/M-phase increased dramatically in hypoxia (Fig. 3. B, D. p<0.05). When exposed to radiation after treatment with SSd, the G2/M arrest was almost completely abolished(Fig. 3. B, D. p<0.05). Meanwhile, in groups of PX-478 plus radiation, a similar cell cycle arrest was induced in hypoxia. In summary, our data here suggested that in hypoxia the radiosensitization of SSd in SMMC-7721 cell was related to the reduction in G2/M-phase population and the increase in G0/G1 arrest, while in oxia the radiosensitization was related to the increase in G0/G1 arrest, but not to the decrease in G2/M phase arrest.

**SSd Elevated Level of p53Expression and Reduced bcl2/BAX Ratio**

In order to reveal whether or not the cell apoptosis induced by SSd, PX-478 or radiation alone and their combination is related with p53-bcl2/BAX pathway, we investigated the level of p53 expression and bcl2/BAX ratio in SMMC-7721 cells in oxia and hypoxia. Western blot analysis revealed that the expression of p53 was up-regulated and bcl2/BAX
ratio was decreased after SSd or radiation alone in oxia and hypoxia, and the differences showed statistical significance (Fig.4. A,B,C. p<0.05). These observations demonstrate that SSd induced cell apoptosis may be related with p53-bcl2/BAX pathway. However, in the case of SSd or PX-478 in combination with radiation, the up-regulation of p53 expression and the reduction of bcl2/BAX ratio were more significant than those in SSd or PX-478 alone(Fig.4. A,B,C. p<0.05). Therefore, at molecular level, SSd potentiates the response of SMMC-7721 cells to radiation. Moreover, in hypoxia, PX-478 alone up-regulated expression of p53 and reduced bcl2/BAX ratio, but these effects not occurred in oxia.

**SSd Altered mRNA Level of p53, BAX and bcl2**

To further confirm whether or not cell apoptosis induced by SSd, radiation and their combinations is related to p53-bcl2/BAX pathway, we analyzed the mRNA levels of p53, bcl2 and BAX in SMMC-7721 cells in oxia and hypoxia. In oxia, except PX-478 alone, the mRNA levels of p53 and BAX elevated, after exposure to SSd or radiation alone and their combinations, but the mRNA levels of bcl2 decreased. Similarly, in hypoxia, the mRNA levels of p53 and BAX increased, while bcl2 decreased in SSd or PX-478 alone and their combinations with radiation (Fig.5. A,B,C. p<0.05, p<0.01). In addition, we also found from these data that all effects occurring in hypoxia are stronger than those in oxia, especially in combined treatment. Thus, these observations confirm that cell apoptosis induced by SSd, PX-478 or radiation and their combinations is related to p53-bcl2/BAX pathway.

**Discussion**

Over the past 20 years, a large number of herbs and other botanical formulations have been constantly developed into radiosensitizers or hypoxic sensitizers, and many of which have already undergone clinical evaluation and showed a favorable effect with safety and lower toxicity [7]. Saikosaponin-d (SSd), an extraction from a type of traditional Chinese herbs, Bupleurum chinensis DC, is reported to have a variety of pharmacological properties including anti-cancer effect [13,14,15]. Here we found that when SMMC-7721 cells were incubated with SSd in either oxia or hypoxia, Cell viability under two conditions were all significantly decreased. In addition, if cells were incubated with SSd prior to radiation, the viability of the cells further decreased at each concentration used. Amazingly, the apoptosis-inducing effects did not occur obviously at 1 µg/ml of SSd in SMMC-7721 cells in either normoxia or hypoxia. However, after treatment with the combination of SSd and radiation, a more significant apoptosis-inducing effect occurred in a dose dependent manner, especially in hypoxic conditions. This implies that SSd has a potential radio-sensitization effect for SMMC-7721 cell in oxia and hypoxia.

In response to radiation-induced DNA damage, replicating cells are known to manifest
as cell-cycle progression delay resulting from the activation of cell cycle checkpoints [20]. Such checkpoints at the G1-S transition and at the G2-M transition are thought to be involved in the detection and correction of DNA damage. The tumor suppressor protein p53 is a critical regulator of the cell cycle checkpoints which can execute the G1/S checkpoint in response to DNA damage and induce a G0/G1 arrest after radiation[21,22,23]. Besides promoting cell cycle arrest, p53 also participates in the activity of oncogene bcl2 family to evoke apoptosis process. Both Bcl2 and Bax are transcriptional targets for p53 protein. The oncogene-derived protein Bcl2 confers negative control in the pathway of cellular suicide machinery. Bcl2 homologous protein, Bax, promotes cell death by competing with Bcl2. While Bax–Bax homodimers act as apoptosis inducers, Bcl2–Bax heterodimer formation evokes a survival signal for the cells, which induces cell cycle arrest or apoptosis in response to DNA damage [24]. Our study found that, in either oxia or hypoxia, levels of p53 and BAX were elevated after their exposure to SSd or radiation alone, and their combinations, but the levels of bcl2 decreased. Hence, it is evident that the cell apoptosis induced by SSd or radiation may be involved in the activation of p53/Bcl2 pathway.

In order to investigate the underlying mechanism of SSd’s radio-sensitization effect on hepatocellular carcinoma cell, in this experiment, we analyzed the alteration of cell cycle using flow cytometry after intervention. The results showed that G0/G1-phase arrest was elicited in SMMC-7721 cells by SSd or radiation alone in either oxia or hypoxia, and further enhanced by the combination treatment of SSd and radiation, which is accompanied by the decrease of S-phase population reflected the activation of a G1/S checkpoint. In addition, radiation alone induced a typical G2/M phase arrest in hypoxia, which was almost completely abrogated by the combination treatment. Furthermore, we also found that cell cycle arrest was companied by the elevation of p53 and BAX in mRNA and protein and decreasing in Bcl2. This may be attributed to the underlying mechanism of radio-sensitization effect of SSd on hepatoma cells.

PX-478, an inhibitor of the hypoxia-inducible factor-1α (HIF1α), has a potent antitumor activity against a variety of human tumor xenografts associated with the levels of the HIF-1α [25]. We found here that PX-478 alone in oxia did not alter the level of p53 expression obviously in SMMC-7721 cell. In hypoxia, however, PX-478 alone could either induce apoptosis and cell cycle arrest or up-regulate the expression of p53. Koh et al [26] agreed that in hypoxia, cell apoptosis induced by PX-478 may contribute to its antitumor activity against HIF-1α expression by increasing p53 level, while in oxia the apoptosis induced by PX-478 does not require oxygen, pVHL nor p53.

**Conclusion**

In summary, the data presented here indicate that SSd can increase the radiosensitivity of hepatoma SMMC-7721 cell and induce the apoptosis of cell, especially in hypoxic condition, the radiosensitizing effect of SSd on hepatoma cells is more obvious, and its
mechanism may contribute to its function to affect Go/G1 and G2/M checkpoints of cell cycle. Thus, it may be a promising radiosensitizer. However, whether or not SSD generates lethal single stranded DNA breaks or reactive oxygen species upon alone or radiation exposure requires further study.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

WBF, DZJ and WXJ designed the research. LS, BMH, MXL and MHB performed the experiments throughout this research. WYL, CYA WXJ and ZY contributed to the reagents, and participated in its design and coordination. WBF, MWL and LS analyzed the data; LS and WBF wrote the paper. Co-first authors: WBF and DZJ. All authors have read and approved the final manuscript.

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Figure 1. Effects of saikosaponin-d and radiation on growth of hepatocellular carcinoma cell SMMC-7721. All dates are presented as mean ± SD. SSd: saikosaponin-d; IR: radiation. *P<0.05, **P<0.01; vs. control.

Figure 2. Effects of saikosaponin-d and radiation on apoptosis in hepatocellular carcinoma cell SMMC-7721. A. Flow cytometry shows apoptotic changes before and after intervention of cell in oxia and hypoxia; B. Apotosis fraction of cell in oxia and hypoxia; SSd: saikosaponin-d; IR: radiation. *p<0.05, **p<0.01; vs. control.

Figure 3. Effect of saikosaponin-d and radiation on cell cycle distribution in human hepatocarcinoma cell line SMMC-7721 in oxia and hypoxia. A. The changes of cell cycle distribution in oxia; B. The changes of cell cycle distribution in hypoxia; C. Statistical analysis showed the changes of the cell cycle before and after intervention in oxia; D. Statistical analysis showed the changes of the cell cycle before and after intervention in hypoxia. SSd: saikosaponin-d; IR: radiation. *p<0.05, ** p<0.01; vs. control.

Figure 4. Effect of saikosaponin-d and radiation on level of p53 and bcl2/BAX ratio in human hepatocarcinoma cell line SMMC-7721 in oxia and hypoxia. A. Western Blotting of p53, Bax and Bcl-2 levels in oxia and hypoxia. B. Relative expression of p53. C. Change of Bax/Bcl-2 ratio. SSd: saikosaponin-d; IR: radiation; *p<0.05, ** p<0.01; vs. control.

Figure 5. Effect of saikosaponin-d and radiation on mRNA level of p53, bcl2 and BAX in human hepatocarcinoma cell line SMMC-7721 in oxia and hypoxia. A. the folds of p53 in mRNA; B. the folds of bcl-2 in mRNA; C. the folds of BAX in mRNA; SSd: saikosaponin-d; IR: radiation; *p<0.05, ** p<0.01; vs. control.
Figure 1

The figure shows the growth inhibition (%) in different conditions. The treatments include Control, SSD(1μg/mL), SSD(1μg/mL) + IR, SSD(3μg/mL), and SSD(3μg/mL) + IR. The conditions tested are Oxia and Hypoxia. The data is presented as bars with error bars, with statistical significance indicated by * and **.
Figure 2

A

![Fluorescence flow cytometry analysis of apoptosis in hypoxic conditions.](image)

- Control
- SSd
- IR
- SSd + IR
- PX-478
- PX-478 + IR

B

![Bar graph showing apoptosis fraction in oxygenated and hypoxic conditions.](image)

- Control
- SSd + IR
- SSd (3μg/mL)
- IR (2GY)
- PX-478
- PX-478 + IR

Figure 2
Figure 3.

A

B

C

D

Figure 3.
Figure 4.
Figure 5.

Figure 5.