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Original Article

Disturbance of redox status enhances radiosensitivity of hepatocellular carcinoma

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Abstract: Aims: High constitutive expression of Nrf2 has been found in many types of cancers, and this high level of Nrf2 also favors resistance to drugs and radiation. Here we investigate how isoliquiritigenin (ISL), a natural antioxidant, inhibits the Nrf2-dependent antioxidant pathway and enhances the radiosensitivity of HepG2 cells and HepG2 xenografts. Results: Treatment of HepG2 cells with ISL for 6 h selectively enhanced transcription and expression of Keap1. Keap1 effectively induced ubiquitination and degradation of Nrf2, and inhibited translocation of Nrf2 to the nucleus. Consequently, expression of Nrf2 downstream genes was reduced, and the Nrf2-dependent antioxidant system was suppressed. Endogenous ROS was higher than before ISL treatment, causing redox imbalance and oxidative stress in HepG2 cells. Moreover, pretreatment with ISL for 6 h followed by X-ray irradiation significantly increased γ-H2AX foci and cell apoptosis, and reduced clonogenic potential compared with cells irradiated with X-rays alone. In addition, HepG2 xenografts, ISL, and X-ray co-treatments induced greater apoptosis and tumor growth inhibition, when compared with X-ray treatments alone. Additionally, HepG2 xenografts, in which Nrf2 was expressed at very low levels due to ectopic expression of Keap1, showed that ISL-mediated radiosensitization was Keap1 dependent. Innovation and Conclusions: ISL inhibited the Nrf2-antioxidant pathway by increasing the levels of Keap1 and ultimately inducing oxidative stress via disturbance of the redox status. The antioxidant ISL possessed pro-oxidative properties, and enhanced the radiosensitivity of liver cancer cells, both *in vivo* and *in vitro*. Taken together, these results demonstrated the effectiveness of using ISL to decrease radioresistance, suggesting that ISL could be developed as an adjuvant radiosensitization drug. Disturbance of redox status could be a potential target for radiosensitization.

Keyword: Redox state, ROS, isoliquiritigenin, radiosensitization, Keap1/Nrf-2

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide [1]. The highest incidences of HCC have been reported in developing countries of East Asia [2]. Surgery and radiotherapy are two commonly used treatment modalities for HCC. About 90% of patients are unresectable at presentation because of tumor size, location, or underlying parenchymal disease [3]. These patients are sometimes recommended for radiotherapy. Conventional radiation such as X-ray radiation and γ-radiation are low LET radiations, which have a restrained efficacy due to the radioresistance of cancer cells [4, 5]. Therefore, studies concerning the radiosensitivity and improvements in X-ray irradiation therapy are a high priority.

As ionizing radiation, X-ray irradiation induces both direct and indirect effects to exposed cells. The direct biological effect involves interaction between photons and DNA, whereas the indirect biological effect is mediated by reactive oxygen species (ROS) produced by radiolysis and subsequent reactions [6]. Radiation-generated ROS is a common mediator of oxidative damage to DNA, biomembrane, and other important cellular structures [7]. These oxida-

tive injuries result in dysfunction of cell organelles, and ultimately lead to cell death or apoptosis [8, 9]. For these reasons, induction of ROS-mediated damage in cancer cells, by pharmacological agents that either promote ROS generation or disable the cellular antioxidant system, are considered potential therapeutic strategies for preferentially killing cancer cells [10].

However, cancer cells are in a state of redox imbalance caused by alterations in the homeostasis of endogenous oxidants and antioxidants [11]. This results in increased oxidants within these cells. Elevated oxidative stress has been found in many types of cancer cells, due in part to their metabolic rate, which is higher than normal cells. To adapt to this oxidative status, many tumor cells possess strong antioxidant defense mechanisms to counteract excessive ROS, maintain redox status, suppress apoptosis and promote growth [12, 13]. The transcription factor nuclear factor erythroid-2-related factor 2 (Nrf-2) is a unique redox regulator that can be modulated in response to redox imbalance caused by oxidative stress [14]. Under normal physiological conditions, Nrf-2 forms an inactive complex with a negative regulator, Kelch-like ECH-associated protein 1 (Keap1), which controls the subcellular localization and steady state levels of Nrf-2. Oxidation or phosphorylation of highly reactive cysteine residues facilitates the dissociation of Nrf-2 from Keap1, with subsequent translocation to the nucleus [15], where it binds to the antioxidant response element (ARE). ARE is a cis-acting enhancer sequence that transcriptionally regulates genes encoding phase II enzymes and antioxidant enzymes, to maintain cellular redox potential and protect against oxidative damage [16]. In cancer cells, high expression of Nrf-2 was involved in regulating the expression of ARE-mediated genes [17, 18] such as NAD(P)H, quinone oxidoreductase 1 (NQO1), catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GSTs), heme oxygenase-1 (HO-1), glutamate cysteine ligase (GCL), and thioredoxin. The products of these genes protect cells against oxidative stress. Because of these mechanisms, most cancer cells survive by neutralizing the effects of oxidative stress, and acquire drug and radiation resistance through the Nrf2-Keap1-ARE signaling pathway [19-21].

ISL is a naturally occurring flavonoid that is nontoxic to humans, and has various biological properties such as antidiabetic, antioxidant, and antitumor activity, as well as vasorelaxant and estrogenic effects [22-26]. Our previous work showed that antioxidant ISL induced oxidative stress by disturbing the redox status and ultimately enhancing the radiosensitivity of HepG2 cells [27]. However, the exact mechanism was not known, especially the effect of ISL on Nrf2-Keap1. The aim of this study was therefore to investigate the prooxidant mechanism of the natural antioxidant ISL, identify the targets of ISL in the Nrf2-Keap1 pathway, and evaluate the effects of ISL on radiosensitization, both *in vitro* and *in vivo*.

Methods and materials

Materials

ISL (purity >99%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). ISL was dissolved in water and filtered through a 0.22 μm filter before use. DFCH-DA was purchased from Sigma Chemical Co. Fetal bovine serum (FBS) were purchased from Hangzhou Sijiqing Biological Manufacture CO., Ltd (Hangzhou, China). All other chemicals were of analytical grade and commercially available.

Cell culture and irradiation

Human liver cancer cell line HepG2 was obtained from China Center for Type Culture Collection (CCTCC), Wuhan, China. Cells were maintained in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin in a humid atmosphere of 5% CO_2 and 95% air at 37°C.

HepG2 cells and were irradiated with 4 Gy X-rays at room temperature. X-rays were given for these cells with a Faxition 43885D X-ray machine operated at 100 kVp energy. The dose rate of X-rays was 1.3 Gy/min.

PCR

Total RNA was extracted from HepG2 cells using the TIANGEN kit (TIANGEN, Beijing, China), according to the manufacturer's instructions. cDNA was synthesized through reverse transcription kit (Takara, Dalian, China) in total volume of 20 μl. The reaction mixture was incubated at 42°C for 50 min and then at 72°C for 15 min. Following this, 1 μl DNA was used for

Table 1. DNA primers used for PCR

Keap-1	5'-CAGATTGGCTGTGTGGAGTT-3' Sense
	5'-GCTGTTCGCAGTCGTACTTG-3' Antisense
Nrf-2	5'-GAGAGCCCAGTCTTCATTGC-3' Sense
	5'-TTGGCTTCTGGACTTGGAAC-3' Antisense
β-actin	5'-TGTTACCAACTGGGACGACA-3' Sense
	5'-TCTCAGCTGTGGTGGTGAAG-3' Antisense

the PCR analyses. PCR was performed using the LA-Taq kit (Takara, Dalian, China) in a 25 μl reaction volume. The sequence information of the primers was listed in **Table 1**. An aliquot of each PCR product was resolved on a 1% vertical agarose gel and electrophoresed in TBE for 1 h. The gels were then digitally photographed and quantified with FluorChem FC2 (Alpha Innotech Corporation, San Leandro, CA).

Western blots

Cells were lysed in RIPA buffer (Beyotime, Nanjing, China). Proteins were separated by 10% SDS-PAGE and transferred to a methanol activated PVDF membrane. The membrane was blocked for 1 h in TBST containing 0.5% FBS and subsequently probed with anti-Nrf2 antibody (Santa Cruz, CA, USA), anti-Keap1 antibody (Santa Cruz), anti-HO1 antibody (Cell Signaling, Danvers, USA) and anti-NQO1 antibody (Cell Signaling) at 4°C overnight with shaking. After 1 h incubation with HRP-conjugated secondary antibody (Santa Cruz), membranes were washed three times for 15 min each with TBST. Reactive proteins were visualized using a chemiluminescence kit (Santa Cruz) according to the manufacturer's instructions. Data were presented as relative protein level normalized to β-actin, and the ratio of control samples was taken as 100%.

Ubiquitination of Nrf2 assay

Cells were lysed in RIPA buffer (Beyotime). Cell lysates were precleared with protein G-agarose (Invitrogen, San Diego, USA) for 1 h at 4°C, followed by incubation with anti-Nrf2 antibody at 4°C overnight with shaking. Immune complexes were precipitated by incubation with protein G-agarose at 4°C for 1 h and a brief centrifugation [28]. The precipitates were washed extensively with PBST and were subjected to fractionation by SDS-PAGE and transferred to a methanol activated PVDF membrane. The membrane was blocked for 1 h in TBST containing 0.5% FBS and subsequently probed with anti-ubiquitin antibody at 4°C overnight. After 1 h incubation with HRP-conjugated secondary antibody (Santa Cruz), membranes were washed three times for 15 min each with TBST. Reactive proteins were visualized using a chemiluminescence kit (Santa Cruz) according to the manufacturer's instructions.

ROS assay

After treatment with 10 μg/ml ISL for different time, the cells were replenished serum-free DMEM with 10 μM of DCFH-DA and incubated for 30 min at 37°C. Then, cells were harvested by trypsin-EDTA solution to produce a single cell suspension. The cells were pelleted by centrifugation and washed twice with PBS to remove the background fluorescence. Stained cells were analyzed using a flow cytometer (Becton Dickinson, Heidelberg, Germany) in combination with Flow Jo software [29].

Confocal microscopy

To visualize the transposition of Nrf2 to nucleus in HepG2 cells, confocal microscopy was used. HepG2 cells were grown in glass-bottom dishes. After different treatments, Cells were fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 10 min. Cells were blocked for 1 h with TBST containing 0.1% BSA followed by a 1 h incubation with rabbit anti-human Nrf2 antibody (Santa Cruz) and mouse anti-human Keap1 antibody (Santa Cruz). The primary antibody was detected with Alexa Fluor-555 goat anti-rabbit secondary antibody (Abcam, MA, USA) and Alexa Fluor-488 donkey anti-mouse secondary antibody (Abcam, MA, USA). The cells were counterstained with DAPI [30].

Confocal images were acquired using a Zeiss LSM-700 confocal microscope equipped with Plan-Apo 63× 1.4 NA oil-immersion objectives. Images were edited with Photoshop (Adobe).

RNA interference

HepG2 cells plated in 60 mm dishes were transfected with Keap1-siRNA, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. siRNA against Keap1 were synthesized by TaKaRa Biotechnology (Dalian, China). These quenches for Keap1-siRNA were 5'-GGCCUUUGGCAUCAUGAACUU-3' (forward) and 5'-GUUCAUGAUGCCAAAGGCCUU-3' (reverse) [31].

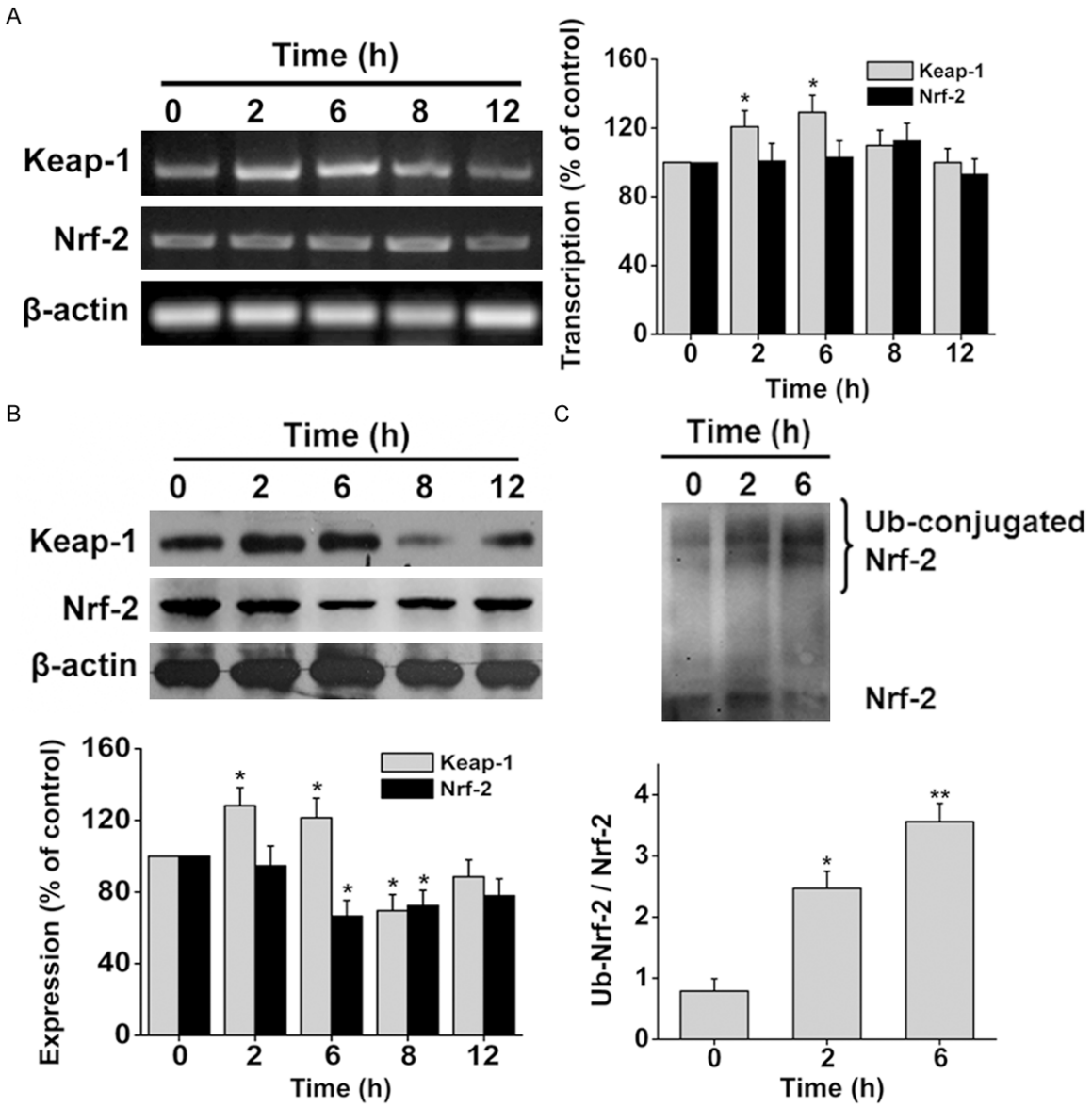

Figure 1. Effects of ISL on Keap-1 and Nrf-2 on HepG2 cells. A. After various times of ISL treatment, expressions of Keap-1 and Nrf-2 mRNA were measured by RT-PCR. β-Actin was used as a standard. B. After various time treatments with ISL, expressions of Keap-1 and Nrf-2 proteins were measured by western blot. C. After various time treatments with ISL, ubiquitination of Nrf2 was measured by immunoprecipitation. All data are expressed as mean ± SEM from three independent experiments. *p<0.05 vs. control group (0 h).

Colony formation assay

HepG2 cells were seeded at 500 cells per 100 mm culture dish and allowed to attach overnight. Cells were treated with 10 μg/ml ISL or left untreated for 6 h, and exposed to 4 Gy of X-ray irradiation. Cells were given fresh medium and maintained under standard cell culture conditions at 37°C and 5% CO_2 in a humid environment. After 8 days, cells were washed twice in PBS, fixed with ethanol, stained with 1% methylene blue, washed with PBS and air dried. The number of colonies was determined by imaging with a Multimage Cabinet (Alpha Innotech Corporation, San Leandro, CA) and using Alpha Ease Fc software.

Detection of apoptosis

Apoptosis was quantified by a combined staining of Annexin V and PI using Annexin V-FITC Apoptosis Detection Kit (BestBio, Shanghai,

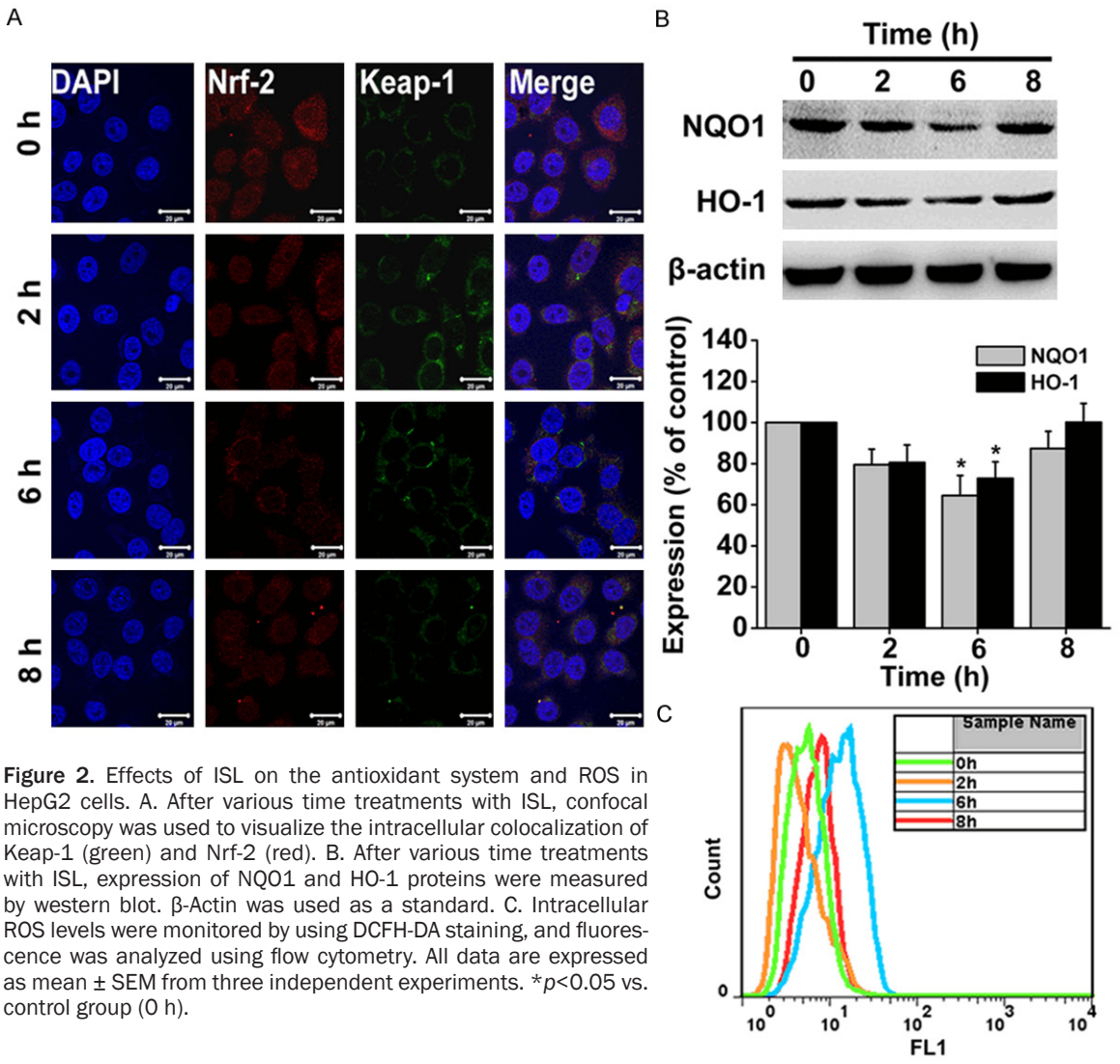

Figure 2. Effects of ISL on the antioxidant system and ROS in HepG2 cells. A. After various time treatments with ISL, confocal microscopy was used to visualize the intracellular colocalization of Keap-1 (green) and Nrf-2 (red). B. After various time treatments with ISL, expression of NQO1 and HO-1 proteins were measured by western blot. β-Actin was used as a standard. C. Intracellular ROS levels were monitored by using DCFH-DA staining, and fluorescence was analyzed using flow cytometry. All data are expressed as mean ± SEM from three independent experiments. *p<0.05 vs. control group (0 h).

China). Cells were prepared according to the manufacturer's instructions. Briefly, approximately 1 × 10^6 cells per experimental condition were harvested, washed with cold PBS twice, and resuspended with 400 μL binding buffer. After adding 5 μL of Annexin V-FITC solution and 10 μL of PI solution, the cells were incubated for 15 min at room temperature in the dark [32]. After the incubation, 10,000 cells were analyzed with the flow cytometer (Becton Dickinson).

In vivo antitumor efficacy

Male athymic BALB/c (nude) mice, 4 weeks of age, were purchased from Institute of Laboratory Animal Sciences, CAMS and PUMC (Beijing, China). To form a tumor xenograft, the mice were subcutaneously injected at the back space with 0.1 ml of cell suspension containing 1×10^7 HepG2 cells. 10 days later, the mice were randomized into four groups (n=5 per group): control, radiation alone, treatment i.p. with ISL (10 mg/kg) alone, and radiation combined with ISL (10 mg/kg, i.p.). Tumors sites were subsequently exposed to a single dose of 4 Gy by a Faxition 43885D X-ray machine. ISL was supplemented 6 h before the radiation exposure. The xenograft tumors were removed and weighted after anatomizing the mice on the 15th day after radiation, and fixed in 10% formalin solution. The tumors were embedded in paraffin and sectioned at 5 μm of thickness. The tumors sections were stained with HE and observed by BX51 optical microscope (Olympus, Tokyo, Japan).

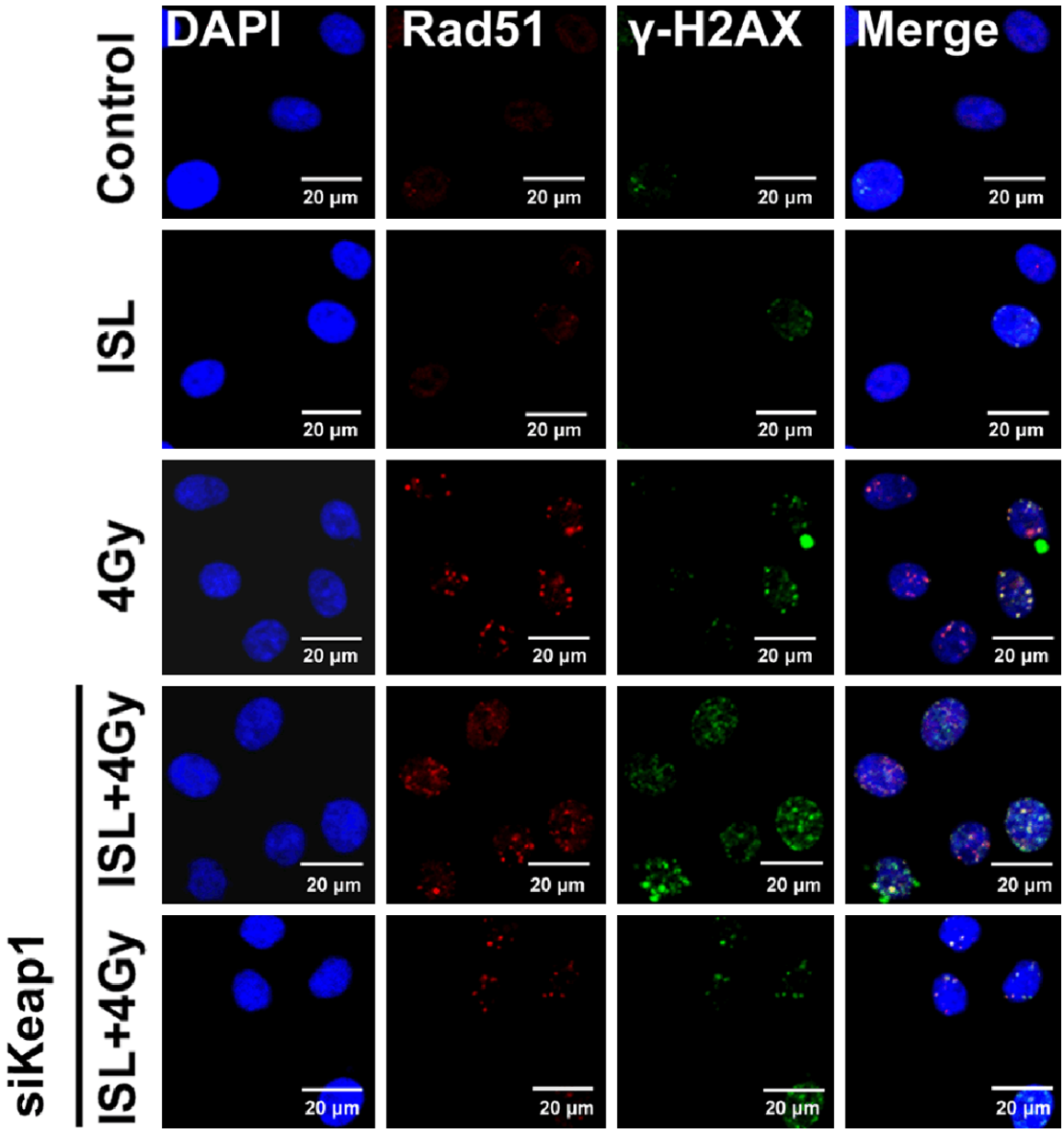

Figure 3. Effects of ISL pretreatment followed by X-ray irradiation on DNA damage of HepG2 cells. Confocal microscopy was used to visualize the intracellular colocalization of γ-H2AX (green) and Rad51 (red) foci. Nuclear staining used DAPI (blue).

Immunohistochemistry for Keap1 and Nrf2

HepG2 cells (1×10^7 cells) were injected into the back space of nude mice. 10 days later, the mice were randomized into two groups (n=5 per group): control, and treatment i.p. with ISL (10 mg/kg) alone. After 6 h treatment with ISL, the xenograft tumors were removed, and fixed in 10% formalin solution. The tumors were embedded in paraffin and cut into 5 μm thick sections. The sections were deparaffinized in three changes of xylene, rehydrated through descending graded alcohols to water and blocked with 5% goat serum for 1 h at 37°C. The specimens were then incubated with rabbit anti-human Nrf2 antibody and mouse anti-human Keap1 antibody at 4°C overnight. The specimens were washed with PBS three times for 3 min each, followed by incubation with HRP-conjugated secondary antibody. Finally, the specimens were incubated with diaminobenzidine (DAB) for 10 min at 37°C and then counterstained with hematoxylin [33].

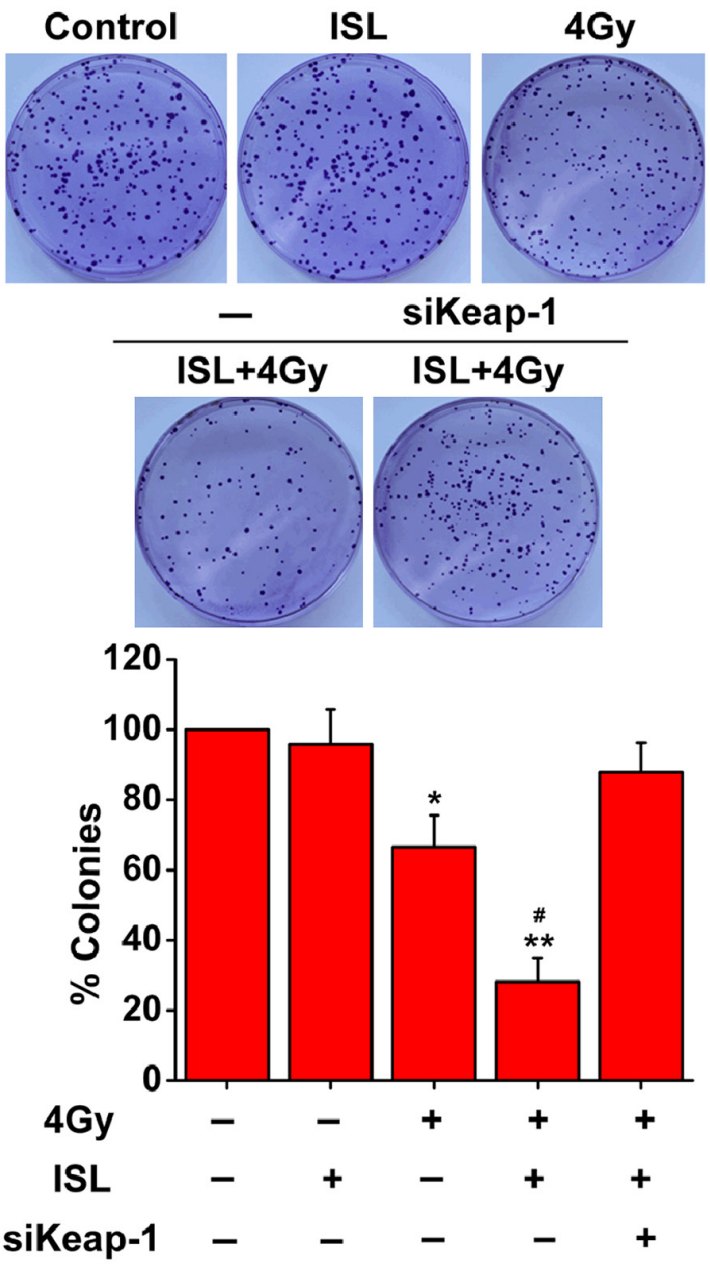

Figure 4. Effects of ISL pretreatment followed by X-ray irradiation on clonogenic potential of HepG2 cells. HepG2 cells were treated with ISL or irradiation alone, or pretreated with ISL for 6 h, followed by irradiation, then allowed to grow for 8 days. All data are expressed as mean ± SEM from three independent experiments. *p<0.05, **p<0.01 vs. control group; #p<0.05 vs. 4 Gy irradiation alone.

Statistical analysis

Data are presented as means ± SEM from at least three independent experiments and evaluated by analysis of variance (ANOVA) followed by Student Newman-Keuls test. Values of P< 0.05 were considered statistically significant.

Results

Isoliquiritigenin enhanced ubiquitination and degradation of Nrf2 through increased Keap1

To determine the transcriptional levels of Nrf2 and Keap1, mRNA expression were measured by RT-PCR. As shown in **Figure 1A**, after different time treatments, 10 μg/ml ISL did not change the mRNA levels of Nrf2 in HepG2 cells, but increased the levels of Keap1 at 6 h. Next, the protein expressions of Nrf2 and Keap1 were determined using western blotting. After different time treatments with 10 μg/ml ISL, the expression levels of Keap1 were enhanced at 6 h. However, Nrf2 levels decreased during the same time period (**Figure 1B**). Nrf2 is mainly found in the cytosol bound to Keap1, which promotes Nrf2 ubiquitination and degradation by a Cul3-E3-dependent mechanism [34]. Ubiquitin-conjugated Nrf2 was enhanced approximately 4.5-fold by ISL treatment at 6 h compared with the control at 0 h (**Figure 1C**). Taken together, these results demonstrated that ISL could indirectly decrease Nrf2 levels through post-translational modulation, which could be related to the upregulation of Keap1.

Isoliquiritigen induced oxidative stress through inhibition of the Nrf2 pathway

Under conditions of stress, Nrf2 can be dissociated from Keap1, then translocated to the nucleus and bound to promoters containing the ARE sequence, activating the transcription of antioxidant enzyme genes. To determine the effect of ISL on the Nrf2-antioxidant pathway, confocal microscopy was used to visualize Nrf2 translocation to the nucleus. As shown in **Figure 2A**, after treatment of HepG2 cells with 10 μg/ml ISL, the signal intensity of Keap1 (green fluorescence) increased, with most immunoreactiv-

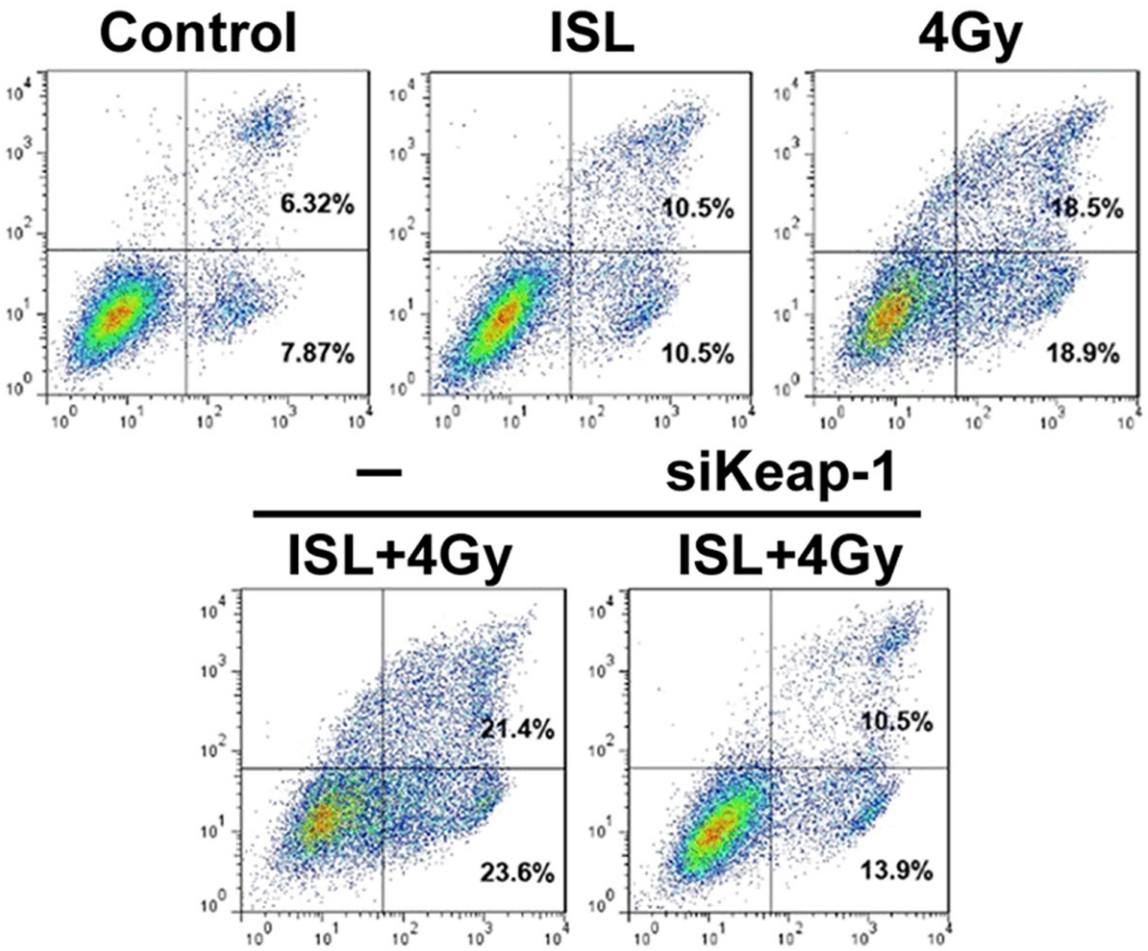

Figure 5. Effects of ISL pretreatment followed by X-ray irradiation on apoptosis of HepG2 cells. HepG2 cells were treated with ISL, irradiation alone, or pretreated with ISL for 6 h, followed by irradiation, then allowed to grow for 24 h. Apoptosis was quantified by combined staining of annexin V and PI, and fluorescence was analyzed using flow cytometry.

ity in the perinuclear region of the cytoplasm. In the same experiments, the signal intensity of Nrf2 (red fluorescence) decreased, particularly at 6 h after ISL treatment caused intranuclear Nrf2 levels to reach their lowest levels. These results demonstrated that ISL could inhibit Nrf2 translocation to the nucleus, which could be related to upregulation of Keap1 and degradation of Nrf2. HO-1 and NQO1 are important downstream proteins of the Nrf2 pathway. **Figure 2B** shows that treatment with ISL resulted in decreased levels of both HO-1 and NQO1; when the ratios of control samples at 0 h were set at 100%, HO-1 and NQO1 were 72.8% and 64.5%, respectively, at 6 h. These results demonstrated that the Nrf2-antioxidant pathway of HepG2 cells decreased to its lowest levels at 6 h after ISL treatment, although endogenous ROS were still generated. Newly formed ROS were generated at a faster rate than ROS metabolism, so the levels of ROS increased at 6 h (**Figure 2C**). Consequently, oxidative stress induced by ISL resulted in a redox imbalance between the excess production of ROS and a defect in the antioxidant defense system.

Isoliquiritigenin pretreatment followed by X-ray irradiation increased DNA damage

The γ-H2AX and Rad51 foci are key events of the DNA damage response, and are considered markers of DNA double-strand breakage (DSB) [35]. **Figure 3** shows the results of γ-H2AX foci in the study of HepG2 cells. When cells were pretreated with ISL for 6 h followed by 4 Gy X-ray irradiation, at 12 h post irradiation, γ-H2AX (green fluorescence) and Rad51 (red fluorescence) foci increased, suggesting that DNA

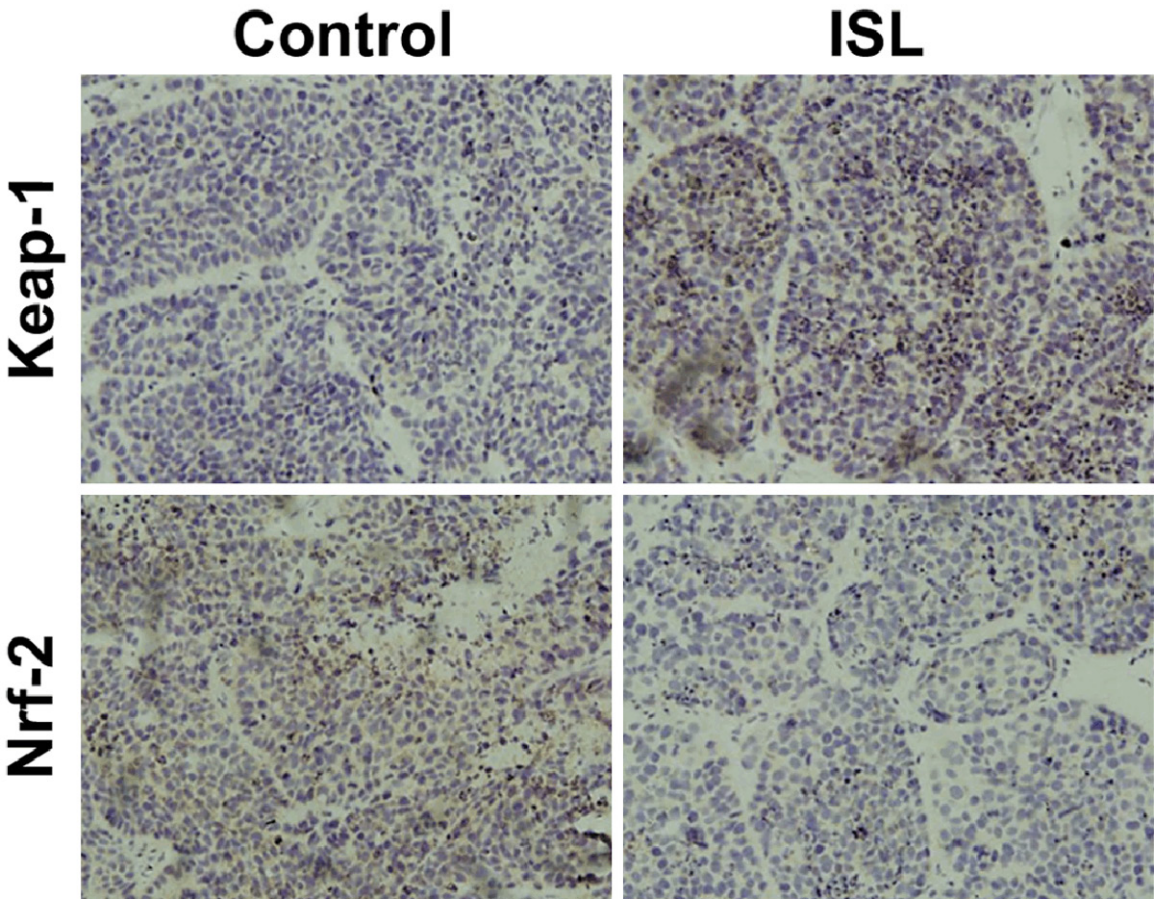

Figure 6. Effects of ISL on Keap-1 and Nrf-2 *in vivo*. Keap-1 and Nrf-2 in HepG2 xenografts were measured by immunohistochemical staining. Photographs were taken with a Olympus BX51 microscope (100×).

damage was exacerbated by ISL. Interestingly, pretreatment with ISL for 6 h, followed by X-ray treatment, did not result in significant DNA damage to Keap1 siRNA-transfected cells (**Figure 3**).

Isoliquiritigenin pretreatment followed by X-ray irradiation reduced the clonogenic potential of HepG2 cells

HepG2 cells were treated with ISL, radiation alone, or pretreated with ISL for 6 h, followed by radiation exposure. The results showed that after 8 days, 4 Gy of X-ray irradiation after 6 h of ISL pretreatment reduced clonogenic potential. The colony forming ratio of cells exposed to X-ray irradiation after 6 h of ISL treatment decreased by 80.5% compared with the control, and decreased by 38.8% compared with 4 Gy X-ray irradiation alone ($^{**}p<0.01$, $^{\#}p<0.05$) (**Figure 4**). These results demonstrated that ISL enhanced the cellular sensitivity to X-ray irradiation; however, the effect of ISL on radiosensitization could be decreased by Keap1 siRNA treatment (**Figure 4**).

Isoliquiritigenin pretreatment followed by X-ray irradiation increased HepG2 cell apoptosis

To further investigate the radiosensitization effect of ISL, apoptosis of HepG2 cells was examined at 12 h after X-ray irradiation, by combined staining of annexin V and PI. At 12 h post-irradiation, the apoptotic rate was approximately 45.1% (early apoptosis, 21.4 ± 1.4%; late apoptosis, 23.6 ± 1.6%) in the 10 μg/ml ISL pretreatment group, in contrast to approximately 37.4% (early apoptosis, 18.5 ± 1.3%; late apoptosis, 18.9 ± 1.5%) in the irradiation alone group (**Figure 5**). In addition, a decrease in levels of apoptosis was detected in Keap1 siRNA-treated cells, compared with the ISL combined X-ray treatment (**Figure 5**). Taken together, the results suggested that ISL pre-

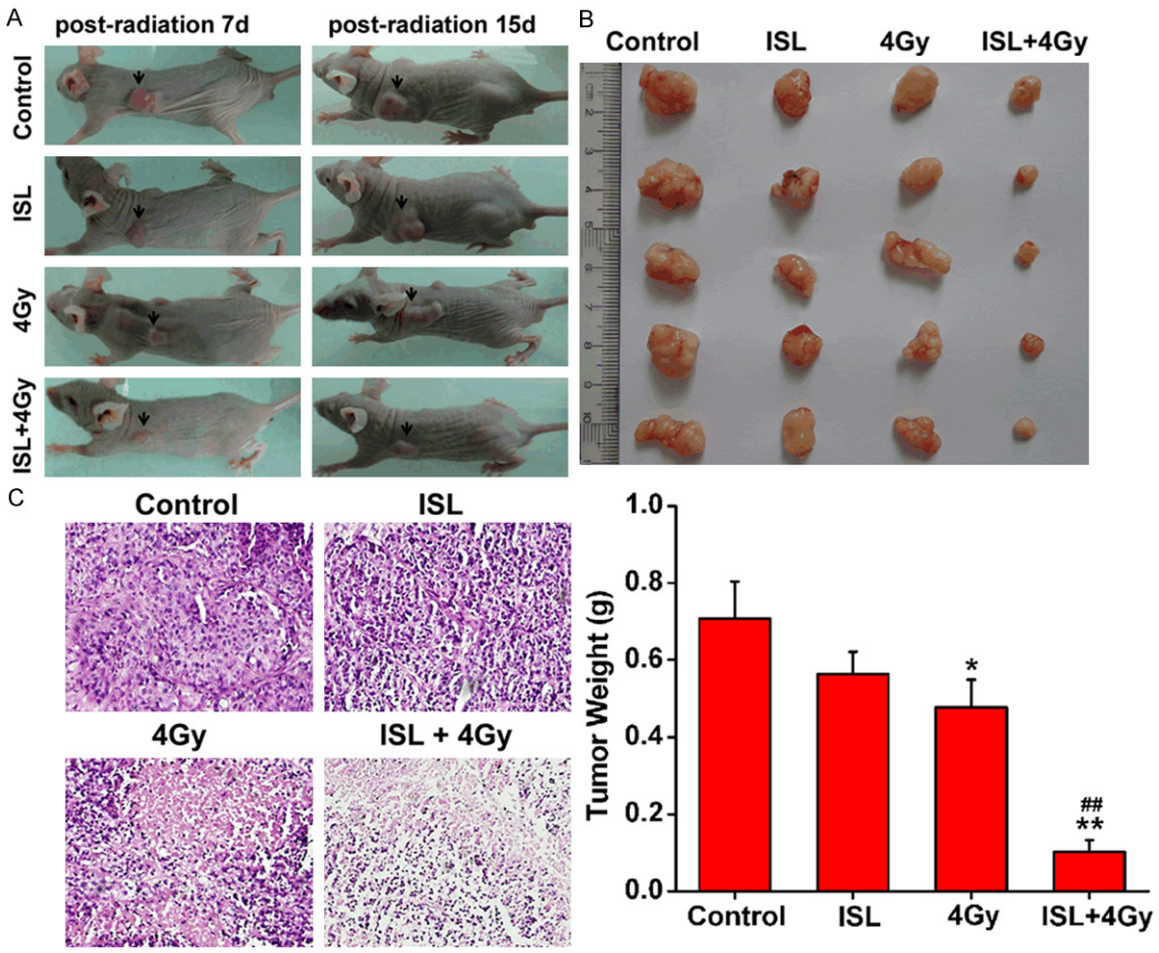

Figure 7. Effects of ISL combined with X-ray irradiation on tumor growth in a HepG2 xenograft model. A. Xenograft mice were photographed at post irradiation 7 days and 15 days. Arrows indicate the tumors sites. B. Tumors were excised and weighed at the end of the experiment (25 days). C. HE staining of tumor tissues in different groups (100×). *p<0.05, **p<0.01 vs. control group; ##p<0.01 vs. 4 Gy irradiation alone.

treatment for 6 h inhibited the Nrf2-antioxidant pathway by increasing Keap1, ultimately inducing oxidative stress in HepG2 cells, and resulting in cancer cells with decreased resistance. Thus, this redox imbalance state enhanced sensitivity to X-ray irradiation in cancer cells, suggesting that Keap1 could be the target for ISL.

Regulation effects of isoliquiritigen on Keap1 and Nrf2 in vivo

To further test the above possibility, we examined the effects of the ISL on the Nrf2/Keap1 pathway using xenograft mouse models. Nude mice were injected with HepG2 cells to induce tumor growth, followed by a single i.p. injection of 10 mg/kg ISL. Tumors were isolated 6 h post injection. Immunohistochemistry data (brown) showed that treatment with ISL for 6 h increased Keap1 positive cells, while Keap1 protein levels were upregulated. At the same time, the number of Nrf2 positive cells decreased when compared with the control group, and Nrf2 protein levels were downregulated (**Figure 6**). These results indicated that ISL could induce disturbance of the redox status, not only *in vitro,* but also *in vivo*.

Radiosensitization effects of isoliquiritigen on xenografts

To record tumor growth, we photographed all mice at post irradiation 7 days and 15 days. As shown in **Figure 7A**, in the control group, tumor growth was not controlled. After treatment with ISL or 4Gy X-ray radiation, continuous growth of tumors was still observed. However, ISL pre-

treatment (10 mg/kg, i.p.) for 6 h, followed by X-ray radiation, inhibited tumor growth. The study was terminated at 15 days after X-ray radiation, and the tumors were collected. The tumor weight was 0.706 ± 0.096 g in the control group, 0.563 ± 0.058 g in the ISL alone group, 0.477 ± 0.071 g in the 4 Gy irradiation alone group, and 0.102 ± 0.031 g in the combination group (**Figure 7B**). Compared with the irradiation alone group, ISL combined with X-ray irradiation inhibited liver tumor growth in nude mice ($^{\#}p<0.05$). In addition, we also used hematoxylin and eosin (HE) staining to evaluate the therapeutic effects of ISL. After radiation treatment, necrosis of the tumor center gradually increased, especially for the combination group (**Figure 7C**). Together, these results were consistent with the radiosensitization effects of ISL.

Discussion

ROS are usually thought to be defensive molecules that destroy exogenous pathogens and act as secondary messengers in signal transduction. However, excess ROS can be deleterious to cellular molecules, including proteins, membrane lipids, and DNA, which could lead to DNA fragmentation and lipid peroxidation [36], resulting in cellular death through necrosis or apoptosis. X-ray, γ-radiation, and other ionizing radiations can promote ROS formation in cells by water ionization, which could result in apoptosis in tumors [37, 38]. Thus, increased production of ROS is an important mechanism during radiotherapy. ISL, a simple chalcone-type flavonoid found in licorice, shallots, and bean sprouts, is reported to have antioxidative effects [39]. Data from the present study showed that 6 h after ISL treatment, intracellular ROS levels were higher in untreated cells ($^{*}p<0.05$) (**Figure 2C**). Our previous work showed that after ISL treatment for 6 h, the ratio of GSH/GSSG was also lower than controls [27]. The antioxidant ISL ultimately induced oxidative stress in HepG2 cells. The prooxidative properties of ISL could therefore result in its radiosensitization, but the reasons for this activity remain unknown.

Antioxidants are defined as substances that, when present at low concentrations compared to an oxidizable compound (DNA, proteins, or lipids), can delay or prevent oxidative damage caused by the presence of ROS [36, 37]. However, some reports have shown that exogenous antioxidants have multiple functions; besides antioxidant activity they can also possess prooxidant activities under certain conditions. It is possible that the prooxidant or antioxidant activities depend on concentration. Recent studies, using cell models, have investigated the prooxidative activity of several polyphenols already identified as antioxidants, such as quercetin, catechins including epicatechin and epigallocatechin-3-gallate (EGCG), and gallic acid [40-44]. Flavonoids (quercetin and fisetin) at low concentrations (10-25 μM) protected rat H4IIE cells against H_2O_2-induced DNA strand breaks and apoptosis, whereas high concentrations (50-250 μM) caused cytotoxicity, DNA oxidative damage, and apoptosis [40]. It was also reported that flavonoids, at high concentrations, can promote ROS generation by autoxidation (e.g., myricetin and quercetagetin) and redox cycling (e.g., quercetin) [44, 45]. In addition to the concentration of antioxidants, the presence of metal ions has also been reported to play an important role. EGCG in the presence of transition metals caused oxidative damage to isolated and cellular DNA [44, 47]. Although almost all these studies emphasized antioxidant properties, the overall activity in biological systems was not thoroughly studied.

Organisms maintain homeostasis by many types of feedback modulations, especially in a cancerous state [48]. The redox status, a key factor in modulating cancer cell proliferation and survival, is controlled by a complex system that includes ROS generation and antioxidant systems such as NADPH oxidase, Nrf-2, CAT, and GSH. Recent studies reported that activation of the Nrf2 pathway mediated the resistance of cancer cells to irradiation [49]. In the present study, we found that intracellular ROS levels in HepG2 cells were significantly downregulated by ISL treatment during early stages (0-6 h), in a time-dependant manner (**Figure 2C**). ISL was assumed to have antioxidant properties. However, treatment of HepG2 cells with ISL for 6 h selectively increased protein levels of Keap1 through enhanced transcription and expression of Keap1 (**Figure 1A** and **1B**). Keap1 effectually induced ubiquitination and degradation of Nrf2 (**Figure 1C**), and inhibited translocation of Nrf2 to the nucleus (**Figure 2A**). Consequently, expression of Nrf2 downstream genes was reduced, and the Nrf2-dependent antioxidant system was suppressed (**Figure 2B**). At same time, endogenous ROS were still being generated in cancer cells. The amounts

of newly formed ROS were greater than those metabolized, so the level of ROS increased at 6 h (**Figure 2C**). Disturbance of redox status could therefore eventually result in oxidative stress. Antioxidant ISL could possess prooxidative properties, and Keap1 could be the target for ISL. With a weakened antioxidant capacity, induction of ROS formation by X-ray irradiation resulted in increased oxidative stress and increased radiosensitivity in ISL-pretreated HepG2 cells, resulting in enhanced radiotherapy effects.

There has recently been controversy over whether supplemental antioxidant administration should be avoided during radiation therapy. Some investigators suggested that antioxidants can remove ROS and protect cancer cells against radiation, so supplemental antioxidants should be discouraged during radiation therapy [50, 51]. Other investigators suggest that antioxidants selectively inhibit repair of radiation damage of cancer cells, but protect normal cells when antioxidants are used before treatment [52, 53]. Since the 1970s, 280 peer-reviewed *in vitro* and *in vivo* studies, including 50 human studies involving 8521 patients, 5081 of whom were given antioxidants, have reported that antioxidants did not interfere with therapeutic treatment modalities for cancer. Furthermore, antioxidants enhanced the killing by therapeutic modalities for cancer, decreased their side effects, and protected normal tissue [54]. The present study showed that the antioxidant ISL possessed a time-dependent dual modulation with regards to redox status. Exposure to X-rays after 6 h treatment of ISL resulted in a radiosensitization effect *in vitro* and *in vivo* (**Figures 3**-**5** and **7**). This reasonable time of antioxidant treatment is an important parameter in radiation therapy. The controversy regarding use of antioxidants during radiation therapy should therefore not be addressed as to whether or not it is to be used, but rather to how it is to be used. ISL might therefore be an effective treatment for radiosensitization, and its effects on the redox status of tumor cells could make it a potential target for radiosensitization.

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Disclosure of conflict of interest

No competing financial interests exist.

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