http://dx.doi.org/10.4314/ajtcam.v12i1.16

SYNERGISTIC EFFECT OF RADIATION AND TRADITIONAL CHINESE MEDICINE RHIZOMA TYPHONII ETHANOL EXTRACTS DEPENDS ON P53 EXPRESSION IN TREATMENT OF LEWIS MOUSE LUNG CANCER CELLS

Yinlong Zhao[1,4], Lingjun Zhao[1], Yunxiao Luo[1], Xin Li[2], Yifan Zhang[3], Xiaodong Liu[4] Yungang Luo[2], Lili Zhong[2]

[1]Department of Nuclear Medicine,[2]Departments of Jilin provincial key laboratory on molecular and chemical genetics, [3]Departments of Thoracic Surgery. The Second Hospital of Jilin University, Changchun 130041, China.
[4]Key Laboratory of Radiobiology (Ministry of Health), School of Public Health, Jilin University, Changchun, 130021, China
***E- Mail**: 13616705@qq.com, centrelab@qq.com
Lili Zhong and Yungang Luo contributed equally to this work.

Abstract

Background: Lung cancer is the leading cause of cancer-related death, and it is the most common cancer in terms of both incidence and mortality. There is an urgent need on novel therapeutic strategies for lung cancer. Traditional Chinese herbal medicines (CHM) have potential valuable for cancer treatment.
Materials and Methods: Lewis mouse lung cancer cell line and Lewis cells tumors xenograft were used in this experiment. MTT assay was used to detect cell proliferation, flow cytometry (FCM) analysis and Western blotting to detect cell apoptosis, and colony formation assay to evaluate the effect of combined therapy of RT+IR.
Results: Our data showed that Rhizoma typhonii (RT) obviously inhibited the proliferations of Lewis cells in time and dose dependent manners by MTT assay and enhanced radiosensitivity by colony formation assay. The effects of RT to Ionizing radiation (IR) therapy were demonstrated radiosensitivity on tumors xenograft experiment. In our study, RT induced apoptotic in Lewis cells directly and enhanced the pro-apoptotic effect of IR by regulating the expression of p53.
Conclusions: These data suggested that RT may be a great potential anti-tumor medicine and the combination of RT and IR may provide a new therapeutic strategy for the treatment of Lewis lung cancer.

Keywords: Rhizoma Typhonii, Radiation, Lung cancer, Combined therapy, Apoptosis

Introduction

The majority of lung cancer patients are not eligible for surgical resection. Ionizing radiation (IR) is one of the most commonly used and efficacious strategies for lung cancer therapies (Pfister et al., 2004). However, there are some limitations in the clinical efficacy of radiotherapy, such as normal tissue tolerance and inherent tumor radio-resistance can hinder successful outcome. Therefore, to develop more effective strategy of side effect is a new challenge" to "to develop more effective strategy with less side effect is a new challenge (Spasova, 2005).

Traditional Chinese herbal medicine (CHM) combined with chemotherapy has shown favorable effects in improving quality of life and prolonging survival on patients with advanced non-small-cell lung carcinoma and has decreased expressions of tumor markers with improving the clinical symptoms and Karnofsky score (Xu et al., 2011; Yan et al., 2011). CHM has some beneficial effects on maintaining immune function and liver protection in patients suffering from chemotherapy (Chan KK, 2011; Liu et al., 2011). The mechanism of anti-tumor effect of CHM includes, but is not limited to regulation of apoptosis (Bakshi et al., 2010; Bajbouj et al., 2012), inhibition of DNA and RNA syntheses (Mousavi et al., 2009).

The CHM Rhizoma Typhonii ethanol extracts (RT) are widely applied to the treatment of tumors (Cao et al., 2011). RT can not only inhibit the tumor growth, but also enhance the patient's immunity (Shan et al., 2001). Although some studies have demonstrated that the combined treatment effects of IR and other chemical anticaxcinogenic drugs were more effective than IR therapy alone (Raben et al., 2005; Ryu et al., 2005; Peng et al., 2008), however, the effect of combined RT with IR was seldom reported.

In the present study, Lewis mouse lung cancer cells and xenograft mice were treated with RT and IR individually or combined for detecting the anti-tumor effects. In addition, the mechanism of enhanced IR sensitivity to lung cancer cells was also investigated.

Methods and Materials

Drug, cell lines and animals

CHM RT powder was purchased from the Affiliated Hospital of Changchun University of Traditional Chinese Medicine (Changchun, Jilin Province, China) and was dissolved in 0.9% NaCl saline prior to intragastric use. Lewis mouse lung cancer cell lines （Departments of Jilin provincial key laboratory on molecular and chemical genetics, The Second Hospital of Jilin University）were cultured in RPMI 1640 medium (Gibco, Invitrogen) (containing 10% fetal bovine serumand 100 μg/ml of both penicillin and streptomycin) at 37°C with 5% CO_2 and digested with 0.25% trypsin for cell passage. Healthy male or female C57BL/6J mice (18 ± 2 g) were purchased from the Laboratory Animal Center of Jilin University. The animal protocols were approved by Jilin University Animal Ethics Committee (No. 2012-046).

Cytotoxicity of RT

Cytotoxicity was detected by MTT assay. Briefly, the cells were treated with 0, 20, 40, 60, 80 and 100 μg/ml of RT in 0.9% NaCl saline at 37°C for 24 h, 48 h and 72 h, respectively. Thereafter, the medium was refreshed and incubated with 0.5 mg/mL of MTT (Sigma St. Louis, MO) for 4 h. Isopropanol was added and optical density was measured at 492 nm using a microplate reader (ELX800,USA).

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IR

IR was performed with Varian 23-ex medical high-energy electron linear accelerator (Varian, USA). Single IR was in range of 0 to 5 Gy at a dose rate of 300 cGy/min. In RT+IR group, 0, 20, and 40 μg/ml of RT in 0.9% NaCl saline was added to the culture medium 1 h prior to IR treatment, respectively. Control cells were kept in a protective box during radiation.

Colony formation assay

Lewis cells (count: 1,000) were cultured in 6-well plates. After 24 h, the cells were treated with RT (0, 20, and 40 μg/ml, respectively). In RT+IR group, IR (0 to 5 Gy) was used 1 h after RT treatment.the cells were cultured for 14 d. Colonies were fixed with 10% formaldehyde and stained with 0.25% crystal violet, followed by colony counting. The fraction of survived colonies in the treatment groups was normalized to the control group. (Franken et al., 2006).

Flow cytometric (FCM) assay

Lewis cells were cultured in 6-well plates at 2×10^5/well and incubated overnight. The cells were treated with 0.9% NaCl saline (control); 20 and 40 μg/ml of RT for 24 h; 3 Gy IR combined with 0, 20, and 40 μg/ml of RT (RT was added 1 h prior to IR treatment), incubated 24 h. Cells were then trypsinized, fixed in ice-cold 70% ethanol and stained with propidium iodide (PI) in phosphate buffer saline (PBS, 0.2 mol/L, pH 7.0). The data were acquired using FACS equipped with Cell Quest Acquisition software (FACS Calibur, Becton Dickinson, USA).

RNA interference (RNAi)

pSuper-p53 RNAi vector (10 μg) and Amphopack (10 μg) plasmid were transiently co-transfected into packaging cell line of 293T (Jilin University Center Laboratory). The pseudoviral particles were purified for 72 h after co-transfection and mixed with polybrene (8 μg/mL). The mixtures were used to infect Lewis cells, and 0.5 μg/ml puromycin was used for selection until positive colonies acquired.

Western blotting analysis

Proteins were extracted by lysis buffer (KeyGen, Nanjing) and concentration was measured using the Nanodrop 1000 Spectrophotometer (Thermo, USA). Protein samples were separated on 13% SDS-PAGE and electroblot onto the Nitrocellulose films (Millipore). Films were incubated in primary antibodies, including anti-Bcl-2, anti-PARP (Cell Signaling Technology, USA), anti-caspase-3, anti-caspase-9 (Santa Cruz, USA) and anti-glyceraldehyde-3-phosphate dehydrogease (anti-GAPDH; Santa Cruz, USA) overnight at 4°C. Blots were washed and incubated for 1 h with goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP second antibodies (Santa Cruz, USA). After another rinse, the film underwent color development by an Enhanced ChemoLuminence (ECL), followed by X-film photography on the UVP EC3 (600) Imaging System (UVP, LLC. Upland, CA, USA). GAPDH protein was used as an inner control.

Tumor Xenografts

C57BL/6J mice were used in tumor xenograft experiment. Briefly, 2×10^6 Lewis cells were injected subcutaneously into the right hind limb. As the tumor size grew to 1 cm^3 approximately, the mice were randomized into five groups（n=5）: control (untreated), RT alone (1.5, and 2.0 mg/kg, once a day until the end of IR treatment), 21 Gy IR alone (3 Gy once a day for 7 d) and IR+RT (1.5 mg/kg + 21 Gy). In IR group,the mice were immobilized in a customized harness and exposed the right hind leg while shielding the remainder of the body by 3.5 cm of lead. Mice were exposed to single 3 Gy IR every day, total dose of 21 Gy. In RT+IR group, RT (1.5 mg/kg) was intragastric administrated to mice 1 h prior to IR treatment, followed by 3 Gy IR. The length and width of tumor diameters were measured using a vernier caliper every 2 d for 15 d, and the tumor sizes were calculated according to formula: Tumor size = $(\text{width})^2\times\text{length}\times0.52$. Xenograft tumors were removed and the sizes were measured as above.

Statistical Analysis

Data were expressed as mean ± SD. Comparisons were made using a one-way ANOVA followed by Dunnett's test. $p < 0.05$ was considered statistically significant. SPSS 13.0 was used for statistical analysis.

Results

RT and IR inhibits Lewis cells

The effect of RT on cells inhibition was detected by MTT assay on Lewis cells treated with various concentrations of RT alone at 24 h, 48 h and 72 h, respectively. After 24 h treatment, 80 μg/mL of RT inhibited Lewis cells compared to the control ($p < 0.05$, Fig. 1A). Cells were significantly inhibited at 72 h treated with 20 μg/mL of RT ($p < 0.05$) and more than 70% after exposure to 100 μg/mL of RT ($p < 0.01$, Fig. 1B). RT alone has obvious cytotoxic effects on Lewis cells in the range of 20 to 100 μg/ml RT in a time-and dose-dependent manner (Fig. 1C). Considering the side effect of RT, lower concentration would be better used for clinical application. Thus 20 and 40 μg/mL concentrations were selected for the following experiment.

To further verify whether RT can enhance the anti-tumor effect of IR on Lewis cells, the cells were performed to examine the survival

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fraction by colony formation assay. Lewis cells were exposed to 0, 20, 40 μg/mL RT for 24 h, treated with 0 to 5 Gy IR. With the increase in both RT concentration and IR dose, cell survival fraction was reduced (Fig. 1D). Our results indicated that there was the synergistic effect of RT and IR on the inhibition of lung tumor. Considering the results of colony formation assay and the routine clinical dose, 3 Gy IR was used for the next experiments to explore the underlying mechanism of synergic effect of RT and IR.

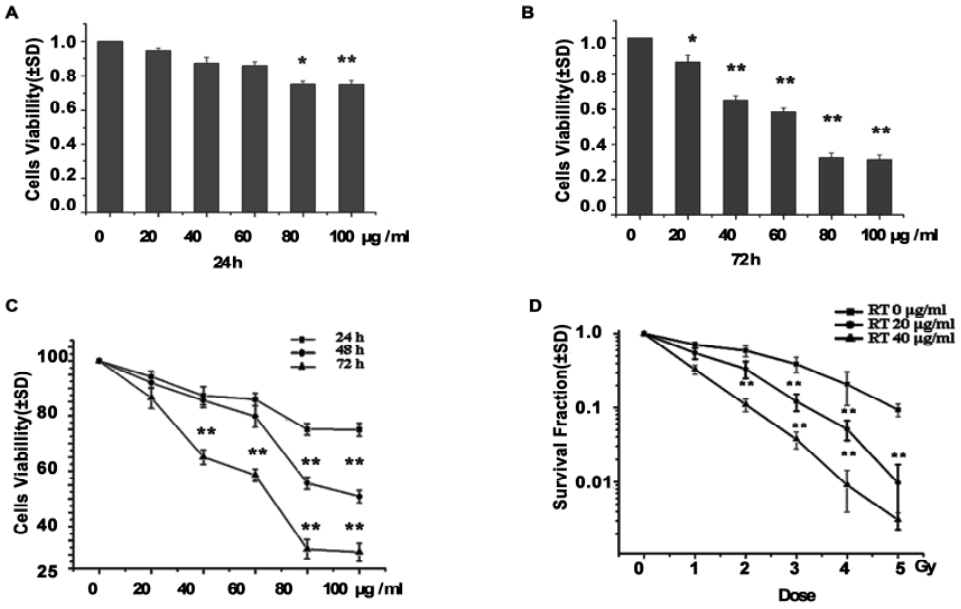

Figure 1: The effects of Rhizoma typhonii on cells viability. (A) The effect of Rhizoma typhonii ethanol extracts (RT) on Lewis cell viability at 24 h by MTT assay. (B) Cell viability analysis on Lewis cells exposed to RT (20-100 μg/ml) at72 h by MTT assay. (C) Cell viability analysis on Lewis cells exposed to RT (20-100 μg/ml) for different time points by MTT assay. (D) the cell radio-sensitivity analysis treated with RT by colony formation assay. Data are represented as mean ± SD (n = 3). Difference comparisons are statistically evaluated using a one-way ANOVA followed by Dunnett's test, and *P < 0.05, ** P < 0.01 indicate statistically significant difference.

RT induces apoptosis directly and enhances the pro-apoptosis effect of IR

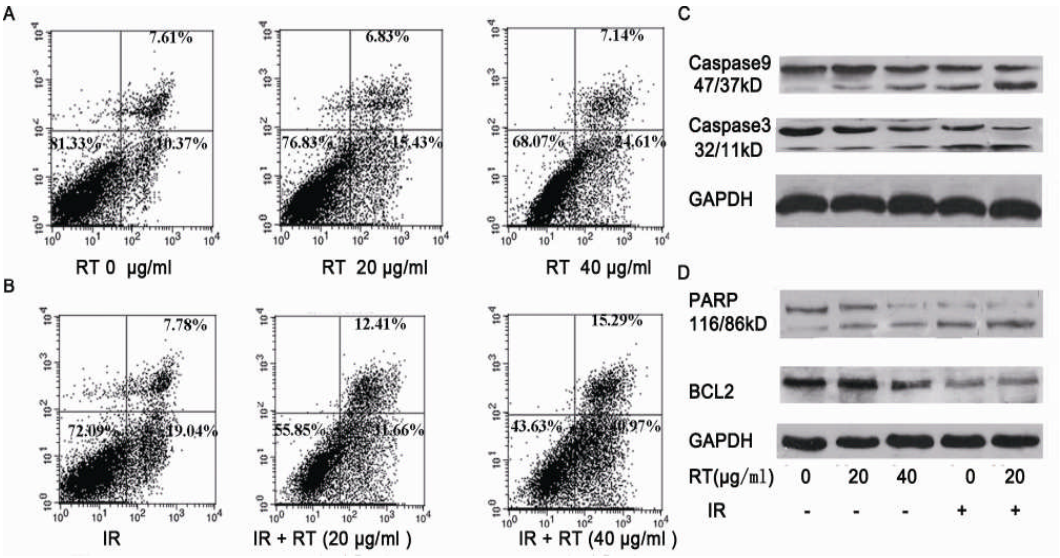

Figure 2: Pro-apoptotic effect of RT. (A) The apoptosis of Lewis cells induced by RT using Flow cytometry. (B) The apoptosis of Lewis cells induced by IR alone or combined with RT (20 and 40 μg/mL) using Flow cytometry. (C) The expression of apoptosis-related proteins treated with RT and/or IR on Lewis cells using Western blotting.

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Lewis cells were exposed to 0, 20, 40 μg/ml RT for 24 h, which were performed to analyse cell apoptosis by FCM (Fig. 2 A, B). The apoptotic rate in the control (untreated) group was 10.37%, however, was 15.43% and 24.61% in 20 μg/mL and 40 μg/mL RT group, respectively. RT enhanced the pro-apoptosis effect treated with IR. IR alone increased the apoptosis rate to 19.04%, and in IR+RT group, (combined with 20 and 40 μg/mL RT treated) the apoptosis rate was up to 31.66% and 40.97%, respectively. These results clearly showed that RT alone could induce cell apoptosis and had a synergetic effect with IR.

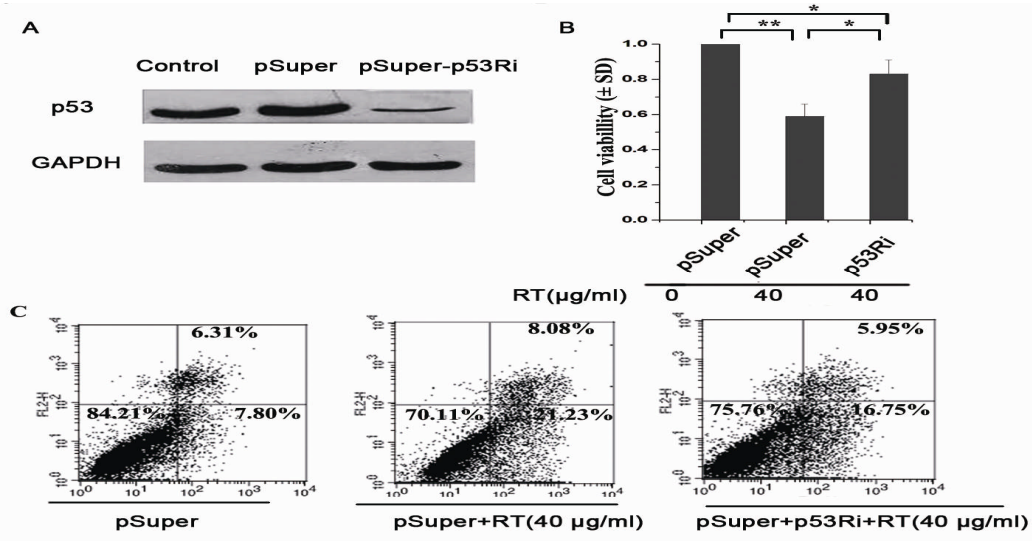

Figure 3: Pro-apoptotic effect of RT depends on p53 gene expression. (A) The expression of p53 protein by Western blotting. (B) Cell viability on p53 gene silencing Lewis cells treated with RT (40 μg/mL) by MTT assay. (C) The apoptosis on p53 gene silenced Lewis cells induced by RT (40 μg/mL) using FCM.

The results showed that the expression of anti-apoptotic protein Bcl-2 decreased, and the expression of pro-apoptotic cleaved-caspase-3, cleaved-caspase-9 and cleaved-poly-ADP-ribose polymerase (PARP) were increased by Western blotting treated with Lewis cells' exposure to RT or IR(3 Gy) (Fig. 3C). It was found that in RT+IR group the expression of Bcl-2 was decreased and the expression of cleaved-caspase-3, cleaved-caspase-9 and cleaved-PARP were increased significantly compared to control group and single treated group.

To further explore the underlying mechanism of pro-apoptotic effect of RT, p53 gene was knocked-down by RNAi technology (Fig. 3A). The results showed that the cell inhibition effect of RT was attenuated after down-regulation of the expression level of p53 gene (Fig. 3B). Meanwhile, the pro-apoptotic effect of RT was also restrained after knocked-down of p53 gene (Fig. 3C).

Combined RT + IR treatment abridged the growth of lung tumor xenograft

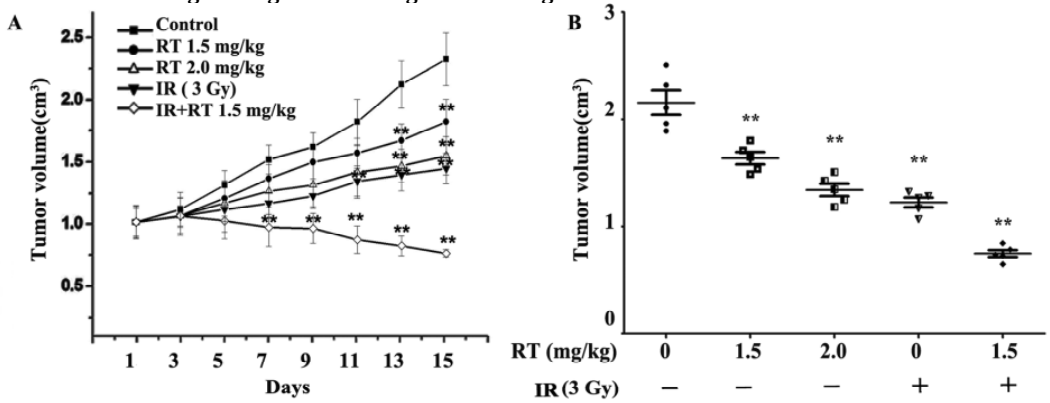

Figure 4: RT inhibits the growth of lung tumor xenograft and enhances IR sensitivity in vitro. (A) Tumor growth curve. (B) Tumors are dissected and the volumes were calculated as described in Materials and Methods. Data are represented as mean ± SD (n = 5).

Lewis cells were injected subcutaneously on the right thighs of C57BL/6J mice. It was performed in five groups treated with RT and IR: negative control, 21 Gy IR alone, RT alone, and RT+IR. The tumor size at the primary site of each group was measured (Fig. 4A). In RT of 1.5 and 2.0 mg/kg, and 3 Gy IR alone groups the tumor size were significantly reduced compared to the control group (Fig. 4A, B). The tumor size in RT+IR group were reduced than RT or IR alone group (Fig. 4B).

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Discussion

Lung cancer is the leading cause of cancer-related death (Ferlay et al., 2010). Lung cancer treatment include surgery, radiotherapy, chemotherapy, and cancer biotherapy are widely applied in treatment strategies. IR therapy is a significant component of cancer treatment for approximately 50% of cancer patients receiving radiotherapy during their course of illness (Baskar et al., 2012). How to improve the sensitivity of tumor cells to IR and decrease the side effect at the same time is urgent issue with the aim to further improve the therapeutic ratio of IR treatment (Brown, 2001; Tofilon et al., 2003; Begg et al., 2011; Ausborn et al., 2012). Therefore, study of IR sensitization will become a hot topic in recent decades (Ferlay et al., 2010). CHM combined with chemotherapy or radiotherapy can prolong survival and improve the quality of life (Tian and Liu, 2010; Xu et al., 2011) and reduce the side effects of radiotherapy, such as decreasing the number of leukocytes, and immune dysfunction (W and Y, 2009).

Rhizoma typhonii is a traditional Chinese herb (Zhang et al., 2007; Zhang et al., 2011). Its ethanol extract—RT has a stimulating activity on human lymphocytes and it could be used potentially for the treatment of cancer. For example, RT has an immune enhancing activity to human T cell and macrophage, through stimulating the killer cell and phagocytosis of tumor cell and allo-antigen, and RT can be used clinically for modulating immune responses and for treating tumor and other diseases(Shan et al., 1999; Shan et al., 2001).

To analyze the toxic effects of RT with various concentrations and at different time points by MTT assay in our study, Lewis cell proliferation was obviously inhibited by 100 μg/mL of RT at 24 h (Fig. 1) and 20 μg/mL RT obviously inhibited Lewis cell proliferation at 72 h. The inhibition was in time-dose dependent manner. Lower concentration of chemo-drug and higher sensitivity of IR are the ideal treatment strategies for cancer therapy. Considering the auxiliary therapeutic role of traditional CHM and the cell inhibition effect of RT, the combined effects of RT and IR should be of clinical significance. The antineoplastic effects of RT combined with IR were demonstrated by colony assay.

As we expect, the sensitivity of IR treatment was enhanced by RT even at low concentration 20 μg/mL RT. In 40 μg/mL RT treatment group, Lewis cells were more sensitivity to IR treatment compared with 20 μg/mL RT treatment group. Our findings indicated that traditional CHM RT has a synergistic effect with IR, and may be used as a complementary measure to enhance the cytotoxicity of IR. Based on the findings, the underlying mechanism of cell inhibition effect and radio-sensitized effect of RT was further explored.

Apoptosis is one of the cell death mechanisms involved in cancer treatment by chemotherapy and IR (Dewey et al., 1995; Mai et al., 2012; Nakayama et al., 2013). The apoptosis of Lewis cells were detected by FCM, which were treated with RT and/or IR. RT could directly induce Lewis cell apoptosis, and the apoptosis rate increased to 15.43% (20 μg/mL RT) and 24.61% (40 μg/mL RT) from 10.37% (untreated control), respectively. Combined RT + IR(3Gy) treatment could enhance the pro-apoptosis effect of IR, up to 31.66% (20 μg/mL RT + IR) and 40.97% (40 μg/mL RT + IR) from 19.04% (IR alone). These results indicate that RT could increase the apoptosis of Lewis cells directly and enhance the pro-apoptosis effect of IR. To examine this proposition, it was performed to detect the apoptosis-related protein expression treated with various concentrations of RT and/or IR on Lewis cells by western blotting. Anti-apoptotic Bcl-2 gene is considered to be involved in resistance to conventional cancer treatment (Fu et al., 2013b; Liu et al., 2013). Caspase-9 is an initiator caspase and aspartic acid specific protease, and has been linked to the mitochondrial death pathway. Caspase-9 is activated during apoptosis and cleaved procaspase-3 which plays a critical role in the execution-phase of cell apoptosis (Perry DK, 1997; Harrington et al., 2008; Arbab et al., 2012). The main role of PARP is to detect and signal the single-strand DNA breaks (SSB) to the enzymatic machinery involved in the SSB repair. PARP also show the ability to induce programmed cell death via the production of PAR, which stimulates mitochondria to release AIF(Yu et al., 2006). Our findings indicated that the expression of Bcl-2 was decreased and cleaved-Caspase-3, cleaved-Caspase-9, and cleaved-PARP were increased treated with RT or IR. Combined RT+IR enhanced these pro-apoptotic effects in Lewis cells. Data analysis from tumor-bearing mice also confirmed that RT has synergistic effect with IR treated. RT or IR treated alone could suppress the Lewis lung cancer cells growth. The tumor sizes of tumor-bearing mice were more significantly suppressed by RT + IR treatment.

Our data revealed that the activation of the apoptosis pathway plays a critical role in RT-sensitized to IR treatment. p53 gene a tumor suppressor is responded to diverse cellular stresses to regulate target genes, which induce cell cycle arrest, apoptosis, senescence, and DNA repair (Smeenk and Lohrum, 2010; Fu et al., 2013a). Previous reports have shown that the pro-apoptosis effect of CHM saffron depends on p53 gene expression (Bajbouj et al., 2012). We have found that p53 gene was knockdown, and p53 silencing attenuated the pro-apoptosis effect and the cell inhibition of RT and RT+IR, indicating the anti-tumor effect of RT is associated with the expression of p53 which is involved in multi-pathways of apoptosis on Lewis lung cancer cells.

Conclusions

Complimentary effect of RT enhanced the cytotoxicity of IR treatment both *in vivo* and *in vitro* Lewis lung cancer cells. The underlying mechanisms of this synergistic effect involved the pro-apoptosis pathways, such as regulation of p53 gene expression. RT may be potential treatment for p53-positve Lewis lung cancer cells.

Author disclosure statement: Publication is approved by all authors; each author has participated sufficiently in submission to take public responsibility for its content. We declare that no actual or potential conflicts of interest are present

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