Knockdown of c-Myc activates Fas-mediated apoptosis and sensitizes A549 cells to radiation

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Abstract. Several studies have demonstrated that cancer radiosensitivity is associated with the deregulation of c-Myc, but the relationship between c-Myc and Fas in radioresistance of lung adenocarcinoma remains unclear. In this study, we established radiation-resistant A549 cell model (A549/R), and investigated the roles of c-Myc and Fas in radiation-induced cytotoxicity of A549 cells. Apoptosis detection showed that there were fewer apoptotic cells in A549/R cells treated with radiation than in A549 cells. Western blotting results demonstrated the inverse expression pattern of c-Myc and Fas in A549 and A549/R cells. Suppression of c-Myc expression by small interfering RNA (siRNA) displayed enhancement of Fas-mediated apoptosis in A549/R cells, accompanying a significant decrease of Bid, Bcl-2, pro-caspase-8, -9 and -3 and increase of Bax. In contrast, Fas-mediated apoptosis was attenuated while Fas expression was suppressed by ectopic expression of c-Myc in A549 cells. Moreover, decreased cell viability and increased induction of apoptosis were observed in A549/R cells followed by combinational treatment of c-Myc siRNA and irradiation, whereas, upregulation of c-Myc reduced the sensitivity of A549 cells to irradiation. These results indicated that c-Myc and Fas regulated the sensitivity of A549 cells to irradiation by regulating caspase-8-mediated Bid activation and the subsequent association with the mitochondrial pathway of apoptosis.

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Introduction

Lung cancer is one of the most serious causes of cancer-related deaths worldwide (1). It affects human life and health intimately, and accounts for 19.4% of all cancer deaths (2). Non-small cell lung cancer (NSCLC) accounts for up to 85% of lung cancers (3-5), which includes adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. The morbidity of lung adenocarcinoma has increased steadily in the past 30-40 years (6,7). The overall 5-year survival rate for a patient with lung cancer is no more than 20%. Approximately 33% of patients with lung cancer are diagnosed at an advanced stage and not suitable for surgery (8-10). Radiotherapy is considered to be one of the most effective and commonly applied treatment to human malignancies. However, radioresistance of tumor cells often become an obstruction in lung cancer treatment. How to reduce the resistance to radiation or improve the radiosensitivity is under active study in lung cancer radiotherapy (11,12).

c-Myc, n-Myc, and l-Myc are the members of the Myc oncoprotein superfamily, they have similar functions, but are expressed differently in various cancer types. c-Myc is usually overexpressed in both blood-borne and solid tumors. n-Myc is mainly expressed in neural tumors, and l-Myc is generally upregulated in small cell lung cancer (13-15). As a heterodimer partner, MYC-associated factor X (MAX) helps c-Myc to regulate at least 15% of gene transcription of human genome (16). c-Myc plays an important role in the broad spectrum of cell functions, including cell proliferation, metabolism,

and differentiation. It can enhance the sensitization of cells to apoptotic stimuli to further promote cell apoptosis (17,18).

Fas signaling pathway is one of the most classical mechanisms inducing apoptosis (19). As a 45-kDa type I transmembrane protein, Fas (CD95, APO-1) is a member of the tumor necrosis factor receptor family (20). Its natural ligand, FasL, is only expressed in the lung, small intestine, testes, and anterior chamber of the eye (21,22). Once FasL combined with Fas to form the death-inducing signaling complex, it initiates caspase-8 cleavage, which activates the downstream effector caspases (e.g., caspase-3), resulting in apoptosis.

Although it has been demonstrated that modulation of FAS and c-Myc altered radiation response in radioresistant cell lines, less is known about the relationship between c-Myc and Fas. In this study, we established radiation-resistant A549 cell model, and investigated the roles of c-Myc and Fas in radiation induced cytotoxicity of A549 cells. We found that inhibition of c-Myc expression had an important role in irradiation-mediated apoptosis in A549/R cells, by upregulation of Fas and activation of caspase-8 which is associated with the Bid-mediated mitochondrial pathway of apoptosis.

Materials and methods

Cell culture and treatment. A549 and LTEP-α-2 cells were obtained from Shanghai Institute of Cell Biology, China. A549/R cells were cultured through X-rays irradiation of up to 68 Gy by using X-RAD 320 (PXi, North Branford, CT, USA) as previously described (23). The cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640; Hyclone; GE Healthcare, Logan, UT, USA) mixing together with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare) at 37˚C with 5% CO_2.

EdU proliferation assay. Cell replication activity was dectected using 5-ethynyl-2-deoxyuridine (EdU) test kits (EdU; RiboBio Co. Ltd., Guangzhou, China) according to the manufacturer's protocol. Briefly, the cells were exposed to 50 μm of EdU for 2 h at 37˚C. Then, the cells were fixed with 4% paraformaldehyde for 20 min and wash with 0.5% Triton X-100 in PBS five times. Afterwards, the cells were reacted with 100 μl of 1X Apollo® reaction cocktail for 30 min. Following wash with PBS, the cells were stained with 100 μl of Hoechst 33258 (5 μg/ml) for 20 min and visualized under a fluorescent microscope (Leica DFC450 C; Leica Microsystems GmbH, Wetzlar, Germany).

Cell cycle analysis. A549, LTEP-α-2 or A549/R cells were seeded in the 6-well plate and irradiated from 0 to 10 Gy 24 h later. Cells were then incubated for 24 h and collected for cell cycle analysis as previous described (24).

Apoptosis detection. The cells were stained with Annexin V-FITC/propidium iodide (PI) (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions as previously reported (23). The cell apotosis was anazlyed using flow cytometry (FACSCalibur; Becton-Dickinson; BD Biosciences, Franklin Lakes, NJ, USA).

MTT assay. Cells were seeded at a density of $1x10^4$ cells/well into a 96-well plate and grown in 5% CO_2 at 37˚C for 24 h. The cells were then transfected with DNA or RNA and their negative controls or irradiated. After transfection or irradiation, each well was added with 10 μl MTT (MTT; Sigma-Aldrich, St. Louis, MO, USA) and incubated with cells for 4 h in an incubator. The formazan was dissolved in 150 μl dimethyl sulfoxide (DMSO) and the optical density was measured using enzyme-linked immunosorbent assay reader (ELx800; USA) at an absorption wavelength of 570 nm.

Transfection. The c-Myc plasmid was purchased from GeneChem, Inc. (Shanghai, China). The siRNA against c-Myc was obtained from GenePharma. Transfection of DNA (plasmids) and RNA (siRNA) was performed using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions.

Western blot analysis. Cells were harvested and lysed as per a previous study (24). The primary antibodies against c-Myc, Fas, Bcl-2, Bax, Bid, caspase-3, caspase-8 and caspase-9 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used according to the manufacturer's instructions, and GAPDH (Bioworld Technology, Inc., St. Louis Park, MN USA) was used as the control. Protein bands were detected by incubation with horseradish peroxidase-conjugated secondary antibody (Beijing Zhongshan Golden Bridge Technology Co., Ltd., Beijing, China) at room temperature for 2 h, and visualized with Fluor Chem FC2 gel imaging system.

Statistical analysis. All experiments were repeated three times independently and the data are presented as the mean ± standard deviation (SD) from triplicate parallel experiments. PRISM (GraphPad Software, San Diego, CA, USA) was performed to analyze the data. Group mean comparisons were compared using an unpaired, two-sided, Student's t-test. ANOVA was used to compare different groups with respect to continuous variables. P-value <0.05 was considered to be significant.

Results

Apoptosis and proliferation changes to radiation in A549/Rcells. To establish radiation-resistant A549 (A549/R) cells, parental A549 cells were prolonged exposed to X-rays for 68 Gy (2 Gy/day, 5 days/week). EdU detection showed fewer cells proliferated in A549/R, A549 and LTEP-α-2 cells treated with 10 Gy X-rays compared with respective control cells (Fig. 1A). Similarly, more cells were blocked in G2-M phase in A549/R and NSCLC cells (Fig. 1B). However, apoptotic detection showed that there were fewer apoptotic cells in A549/R cells treated with radiation than that in A549 and LTEP-α-2 cells (Fig. 1C). MTT assay further proved that the number of viable cells was much more in the A549/R cell cultures exposed to 10 Gy of X-rays than those in A549 and LTEP-α-2 cells (Fig. 1D). These data suggested that the resistance of radiation-induced apoptosis is involved in A549/R cells compared with NSCLC A549 and LTEP-α-2 cells.

Resistance of A549/R cells to radiation involves c-Myc and Fas. Next, we studied whether c-Myc and Fas play important

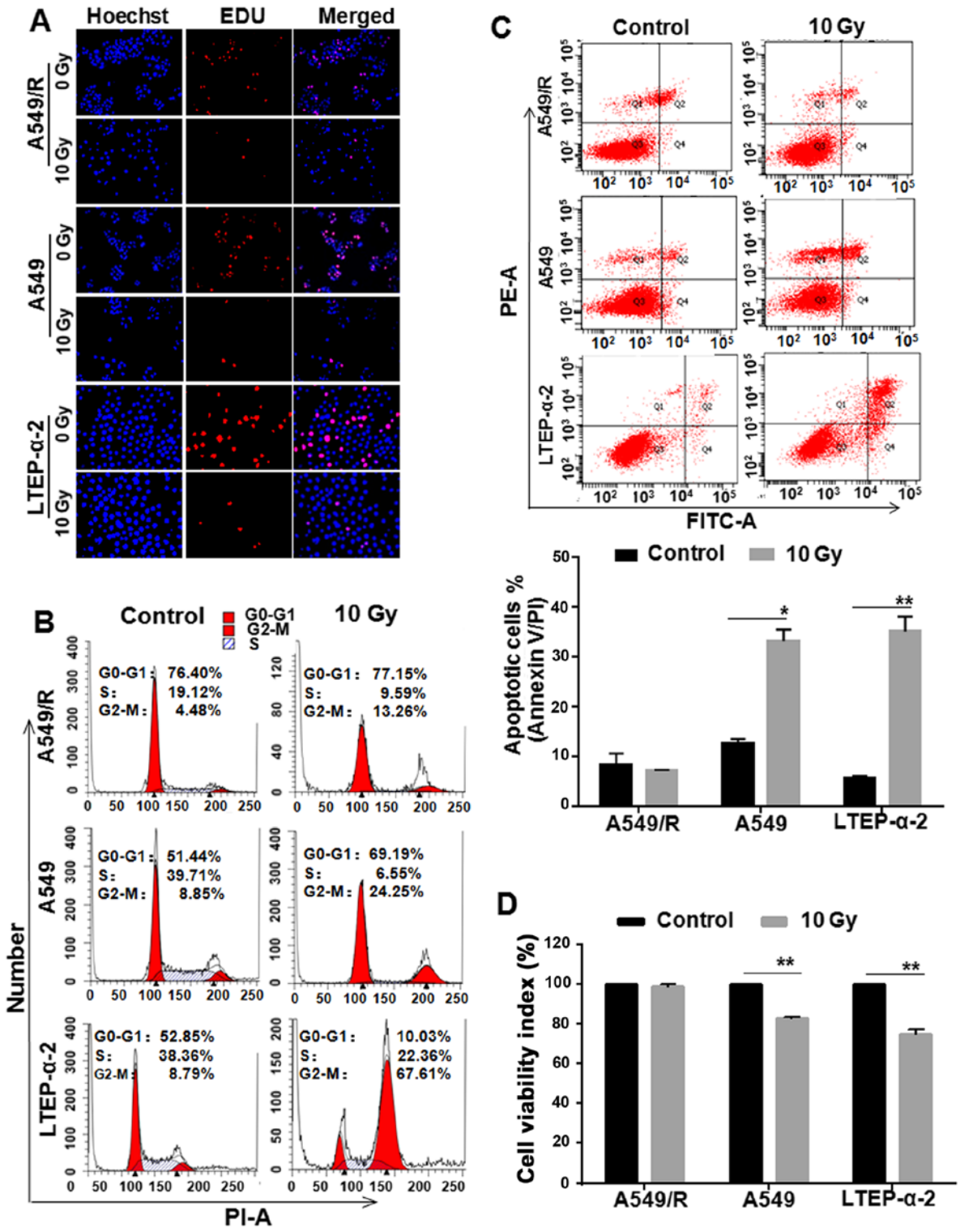

Figure 1. Apoptosis and proliferation changes to radiation in A549/R, A549 and LTEP-α-2 cells. (A) EdU analysis of cell proliferation. EdU detection showed fewer cells proliferated in A549/R, A549 and LTEP-α-2 cells treated with 10 Gy X-rays compared with respective control cells. Hoechst (blue) was used to stain nucleus and EdU (red) showed incorporated cells. Bar, 50 μm. (B-D) A549/R, A549 and LTEP-α-2 cells were irradiated to 10 Gy. Cells were then incubated for 24 h and collected for cell cycle (B), apoptosis (C) and cell viability assay (D). *P<0.05 and **P<0.01, vs. control, respectively.

roles in the resistance of A549/R cells to irradiation, both of which have been implicated in tumor aggression and radiation therapy (25,26). Intriguingly, Fas expression notably decreased in A549/R cells compared to A549 cells, while c-Myc was highly expressed (Fig. 2A), indicating that there is an inverse expression pattern between c-Myc and Fas in A549 and A549/R cells.

Only the Fas ligand (FasL), binds to their cell surface death receptor Fas, the extrinsic pathway of apoptosis is activated. To determine if FasL was involved in the radioresistance of A549/R cells, we used the exogenously sufficient FasL to activate the Fas-meditated signals. We found that 25 ng/ml FasL significantly inhibited A549 cell growth compared with

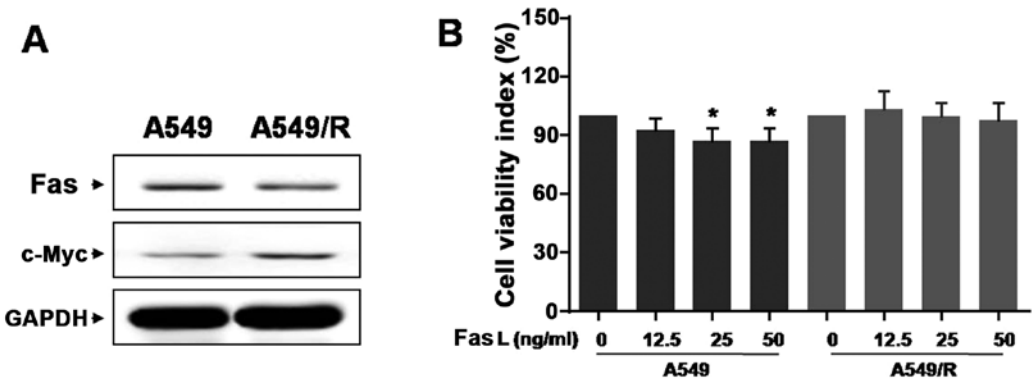

Figure 2. Resistance of A549/R cells to radiation involves c-Myc and Fas. (A) Western blot analysis of Fas and c-Myc expression in A549 cells and A549/R cells. GAPDH was used as loading control. (B) Cell viability was analyzed by MTT assay. A549 and A549/R cells were treated with different doses of FasL (0, 12.5, 25 and 50 ng/ml) for 24 h, then MTT assay was performed. *P<0.05, vs. untreated control.

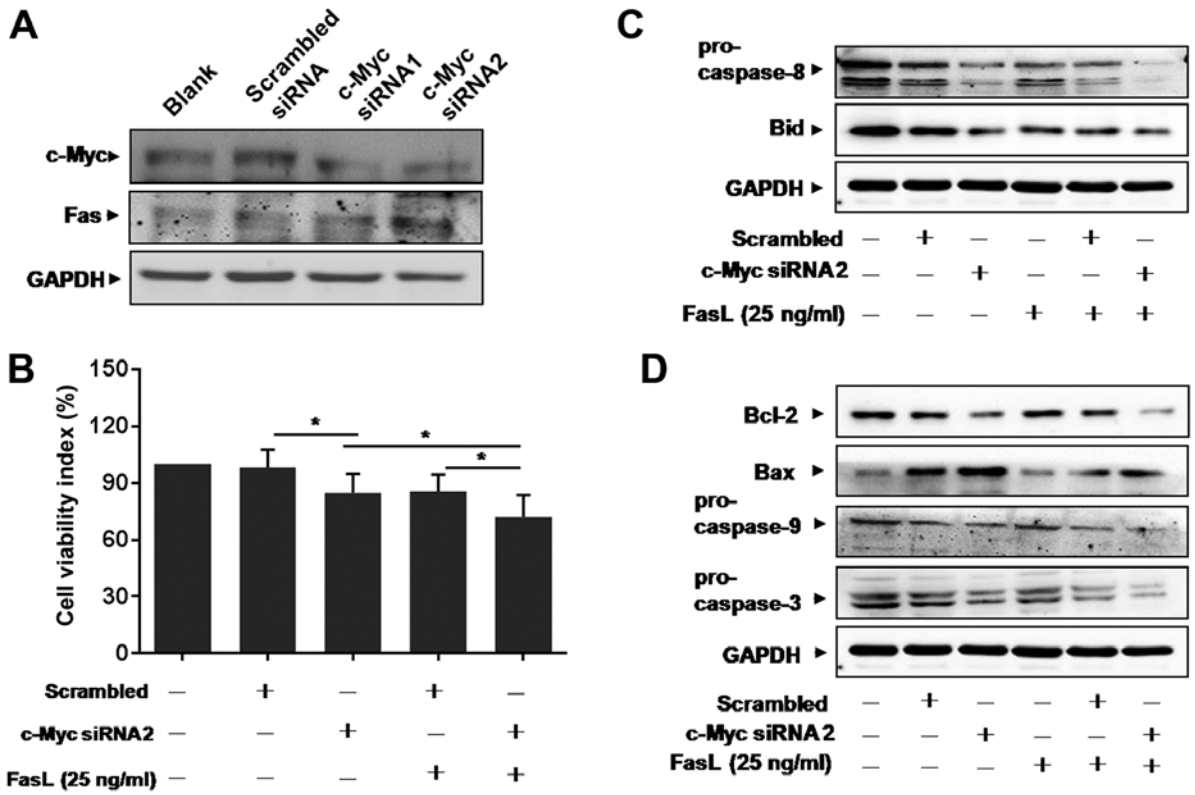

Figure 3. Knockdown of c-Myc enhances Fas-mediated apoptosis. (A) Western blotting was used to detect c-Myc and Fas expression in A549/R cells transfected with Scrambled siRNA or c-Myc siRNA for 24 h. (B) Cell viabilities were measured by MTT assay after A549/R cells were treated with c-Myc siRNA alone or combined with FasL (25 ng/ml) for 24 h. *P<0.05, vs. untreated control respectively. (C and D) Western blotting was used to analyze the expression of pro-caspase-8, Bid, Bcl-2, Bax, and pro-caspase-9 and -3 in A549/R cells treated with siRNA alone or combined with FasL (25 ng/ml) for 24 h. GAPDH was used as loading control.

untreated cells. Nevertheless, 25 ng/ml FasL could not obviously suppress A549/R cell growth (Fig. 2B). Such finding suggests that A549/R cell radio-resistance is associated with Fas downregulation rather than FasL.

Knockdown of c-Myc enhances Fas-mediated apoptosis. Little is known about the relationship between c-Myc and Fas in lung cancer up to now. We supposed that c-Myc could affect Fas signals and designed siRNA specific to c-Myc to transfect A549/R cells. The efficacy of siRNA to inhibit c-Myc is shown in Fig. 3A. After transfection, the expression level of Fas increased in c-Myc siRNA-transfected cells, compared with scrambled siRNA-transfected cells (Fig. 3A). MTT results showed that the number of viable A549/R cells decreased in c-Myc siRNA treated cultures (Fig. 3B). We found that FasL treatment further decreased the number of viable cells in c-Myc siRNA-transfected A549/R cells (Fig. 3B).

Caspase-8 is a major initiator caspase in the death receptor-dependent apoptotic pathway and a known mediator of Bid (27). Our results showed that the expression of pro-caspase-8 and total Bid decreased in c-Myc siRNA-transfected A549/R

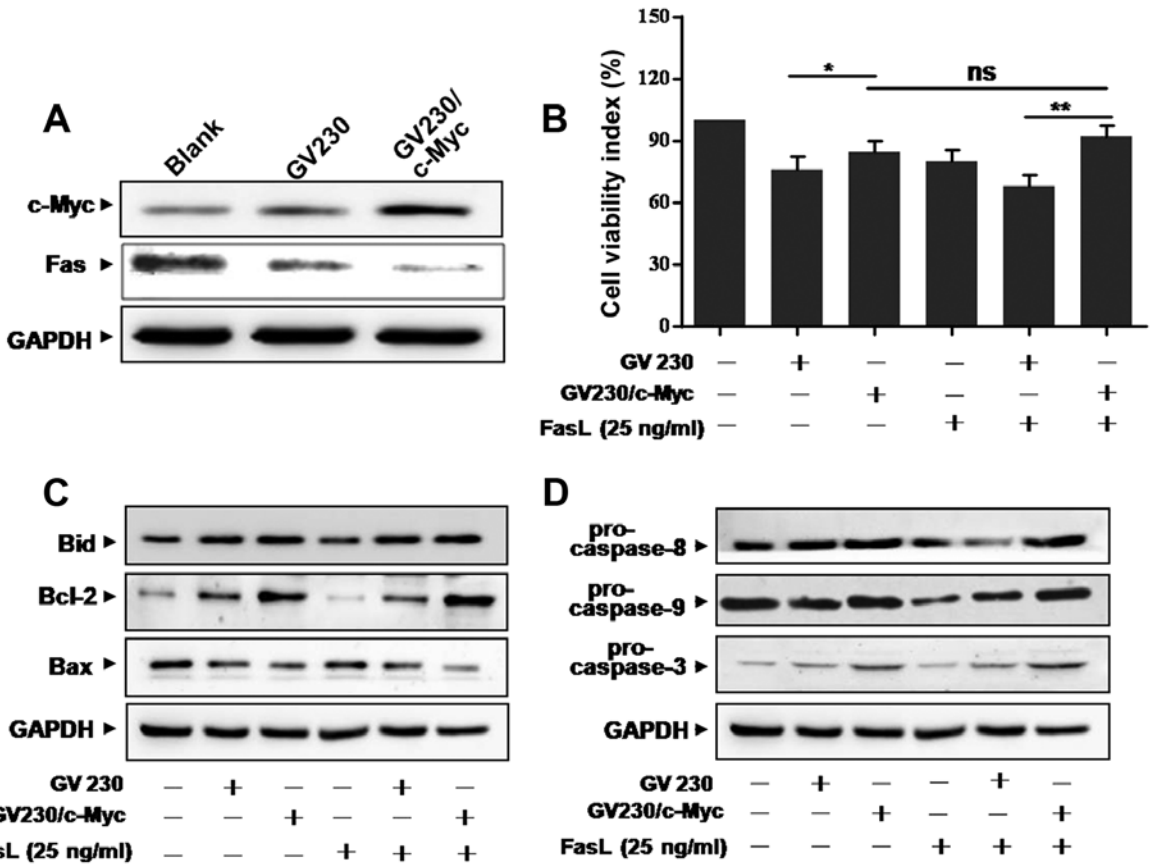

Figure 4. Overexpression of c-Myc attenuates Fas-mediated apoptosis. (A) Western blotting was used to detect c-Myc and Fas expression in A549 cells transfected with GV230 or GV230/c-Myc overexpression plasmid for 24 h. GAPDH was used as loading control. (B) Cell viabilities were measured by MTT assay after A549 cells treated with GV230/c-Myc alone or combined with FasL (25 ng/ml) for 24 h. $^{*}P<0.05$ and $^{**}P<0.01$; ns; no significant differences. (C and D) Western blotting was used to analyze the expression of Bid, Bcl-2, Bax, pro-caspase-8, -9 and -3 in A549 cells treated with GV230/c-Myc alone or combined with FasL (25 ng/ml) for 24 h. GAPDH was used as loading control.

cells (Fig. 3C). FasL treatment further decreased the levels of pro-caspase-8 and total Bid (Fig. 3C). Furthermore, Bid is known as a critical mediator of the mitochondrial pathway of apoptosis following death receptor activation (27). We next investigated the expression of anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax, both of which are critical mediators of the mitochondrial pathway of apoptosis. Confirming our hypothesis, Bcl-2 expression decreased, while Bax increased in cells transfected with c-Myc siRNA (Fig. 3D). The expression of pro-caspase-9 and -3 was also downregulated significantly in c-Myc siRNA-transfected A549/R cells (Fig. 3D), which lead to increase in cell apoptosis. These results indicate that Fas-mediated apoptosis is required for radiation induced apoptosis in A549/R cells.

c-Myc overexpression attenuates Fas-mediated apoptosis. To further investigate the effects of c-Myc on Fas-mediated apoptosis in A549 cells, c-Myc overexpression plasmid (GV230/c-Myc) was employed. First, the overexpression efficiency was confirmed by western blotting (Fig. 4A). Interestingly, the expression level of Fas was attenuated by ectopic expression of c-Myc in A549 cells (Fig. 4A). MTT assay showed that the reduced number of viable cells by FasL was further attenuated by overexpression of c-Myc in both GV230/c-Myc- and FasL-treated A549 cells (Fig. 4B). We then determined whether overexpression of c-Myc could affect Fas-mediated apoptosis pathway. Results showed that the levels of Bid and Bcl-2 were upregulated, while Bax was downregulated in GV230/c-Myc-transfected A549 cells treated with FasL (Fig. 4C). Also, the levels of pro-caspase-8, -9 and -3 increased in GV230/c-Myc-transfected A549 cells (Fig. 4D). These results supported that upregulation of c-Myc resulted in decreasing Fas expression level, which is related to Fas-mediated apoptosis.

Knockdown of c-Myc sensitizes A549/R cells to radiation. We supposed that downregulation of c-Myc could sensitize A549/R cells to radiation. To address this hypothesis, we treated A549/R cells with c-Myc siRNA for 24 h before subjecting to radiation. MTT assay showed that cell viability markedly decreased in cells transfected with c-Myc siRNA compared with scrambled siRNA when A549/R cells were exposed to FasL alone or combined with 10 Gy of X-rays (Fig. 5A). Apoptosis assay showed that more apoptotic cells were found in c-Myc siRNA transfected A549/R cells exposed to radiation (Fig. 5B). These results indicated that knockdown of c-Myc significantly enhanced the sensitivity of A549/R cells to radiation.

We further evaluated the potential mechanisms of c-Myc siRNA on sensitivity of A549/R cells to radiation. When c-Myc expression decreased after siRNA treatment, Fas was upregulated, especially in 10 Gy of X-ray-treated

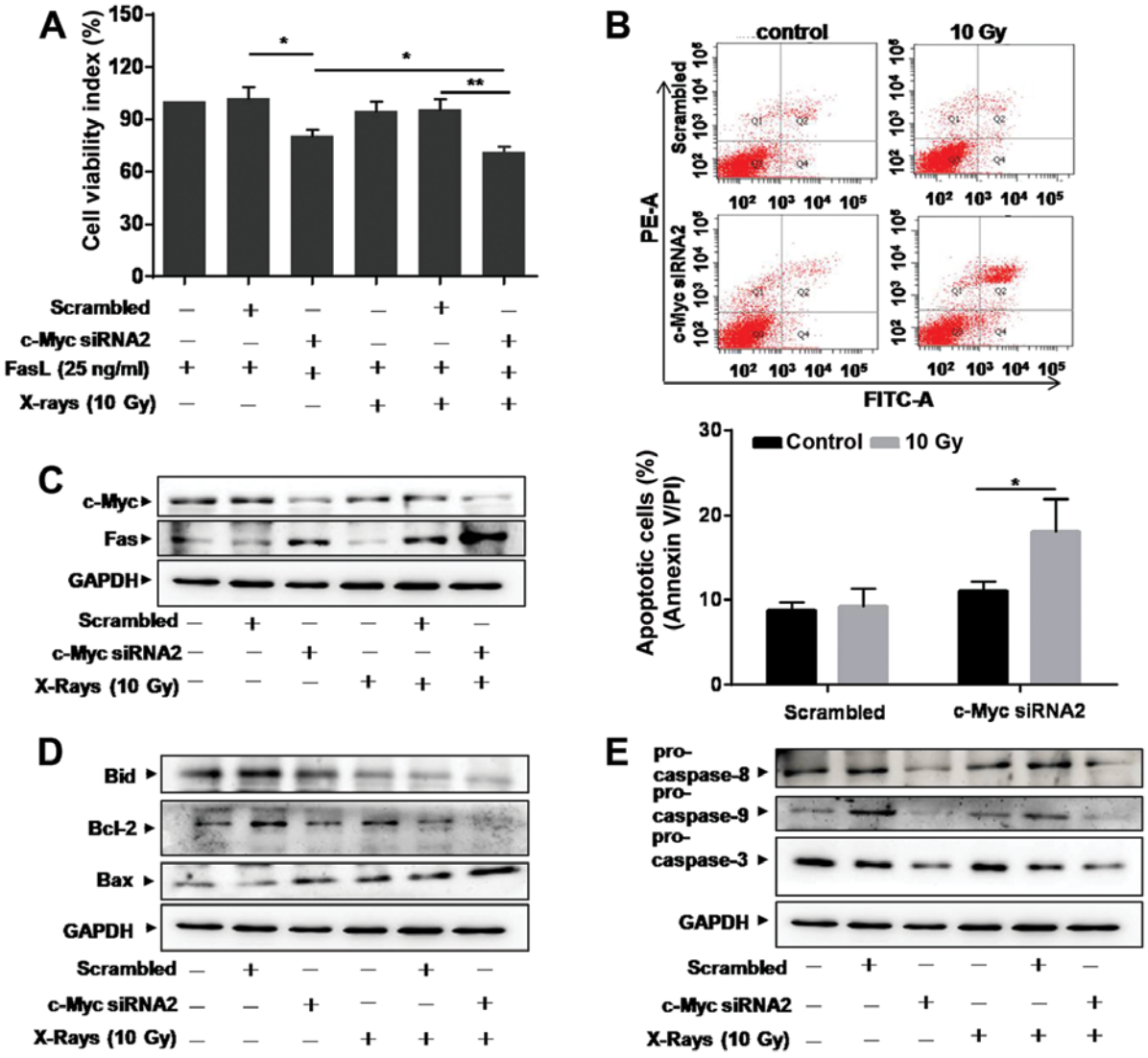

Figure 5. Knockdown of c-Myc sensitizes A549/R cells to radiation. (A) MTT assay was performed to detect the cell viability of scrambled siRNA or c-Myc siRNA-transfected A549/R cells after FasL or radiation treatment alone or together. $^{*}P<0.05$ and $^{**}P<0.01$, vs. untreated control respectively. (B) Apoptosis of scrambled siRNA or c-Myc siRNA-transfected A549/R cells was determined using flow cytometry after radiation treatment. $^{*}P<0.05$, vs. control. (C-E) Western blotting was performed to detect the expression of Fas, c-Myc, Bid, Bcl-2, Bax, pro-caspase-8, -9 and -3 in scrambled siRNA or c-Myc siRNA-transfected A549/R cells treated with radiation. GAPDH was used as loading control.

cells (Fig. 5C). Simultaneously, total Bid and anti-apoptotic protein Bcl-2 decreased, while Bax was upregulated in c-Myc siRNA-transfected A549/R cells (Fig. 5D). Western blotting demonstrated that the levels of pro-caspase-8, -9 and -3 were decreased, which are related to Fas-meditated apoptosis (Fig. 5E). These results provided evidence for the mechanisms of c-Myc and Fas in the sensitivity of A549/R cells to radiation.

Upregulation of c-Myc reduces the sensitivity of A549 cells to radiation. To further investigate the effects of c-Myc deregulation on lung cancer cells to irradiation. The plasmid of GV230/c-Myc or GV230 was transfected into A549 cells, respectively. Compared with that of GV230-transfected cells, the cell viability significantly increased in GV230/c-Myc-transfected A549 cells when exposed to FasL (25 ng/ml) or irradiation (10 Gy) (Fig. 6A). Likewise, apoptosis of GV230/c-Myc-transfected A549 cells was significantly decreased compared with GV230-transfected cells when treated with radiation (10 Gy) (Fig. 6B). These data indicated that the expression level of c-Myc played a critical role in radioresistance of A549 cells.

Next, western blot assay was performed to detect the expression of Fas. As shown in Fig. 6C, when combined with irradiation treatment (10 Gy), c-Myc overexpression significantly decreased Fas expression compared with GV230 group. The expression of Bid and Bcl-2 increased obviously in GV230/c-Myc-transfected A549 cells compared with the cells transfected with GV230 after exposure to 10 Gy of X-rays, but the expression of Bax decreased (Fig. 6D). Also, western blotting results indicated that the expression of pro-caspase-8, -9 and -3 protein in c-Myc overexpression A549 cells increased in comparison with those derived from GV230-transfected A549 cells, when treated with radiation (Fig. 6E). Collectively, upregulation of c-Myc could lead to the decreased radiation-induced Fas-mediated apoptosis in A549 cells.

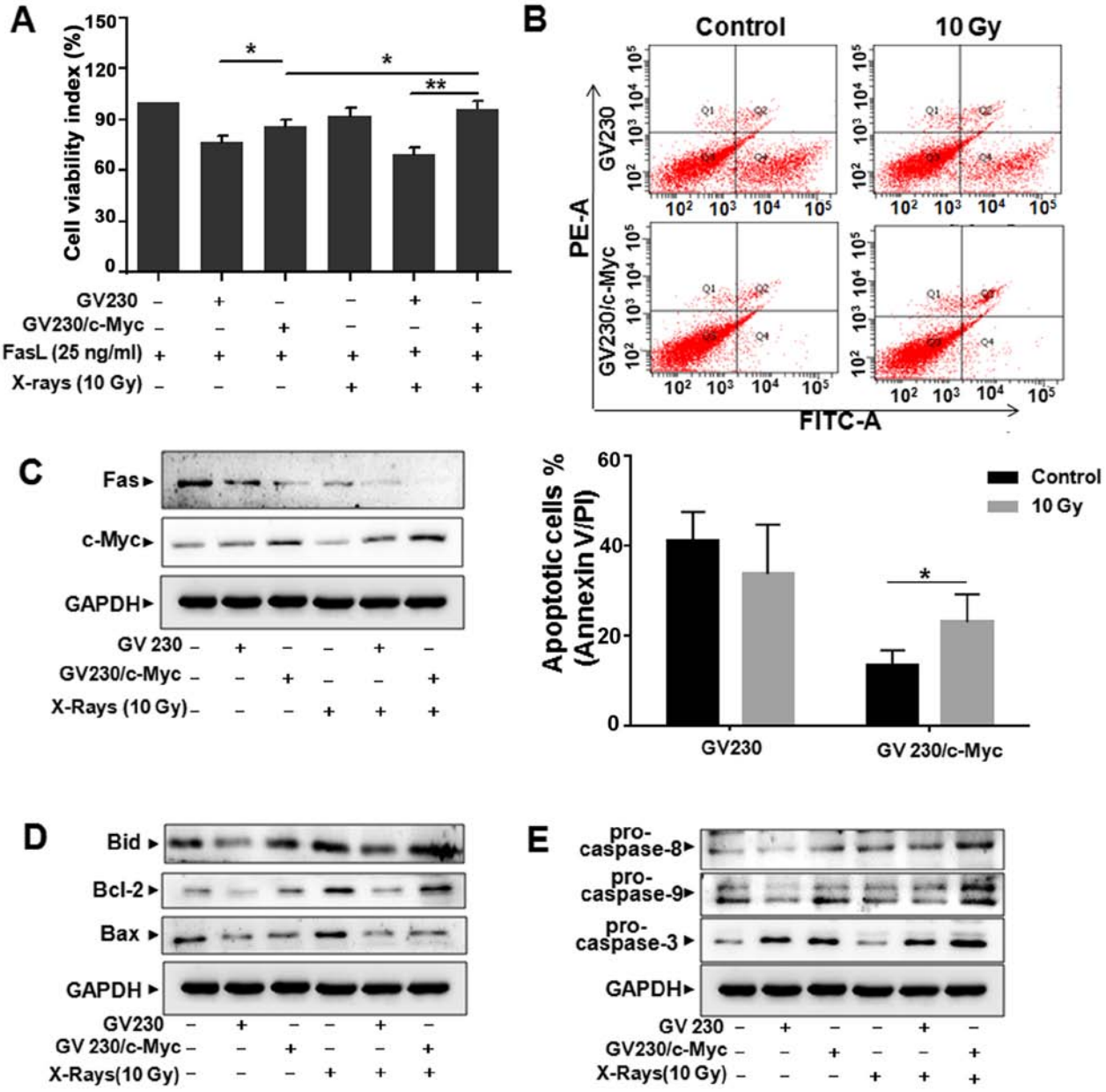

Figure 6. Upregulation of c-Myc reduces the sensitivity of A549 cells to radiation. (A) MTT assay was performed to detect the cell viability of GV230 or GV230/c-Myc-transfected A549 cells after FasL or radiation treatment alone or together. *P<0.05 and **P<0.01, vs. untreated control respectively. (B) Apoptosis of GV230 or GV230/c-Myc-transfected A549 cells was determined using flow cytometry after radiation treatment. *P<0.05, vs. control. (C-E) Western blotting was performed to detect the expression of Fas, c-Myc, Bid, Bcl-2, Bax, pro-caspase-8, -9 and -3 in GV230 or GV230/c-Myc-transfected A549 cells treated with radiation. GAPDH was used as loading control.

Discussion

A series of reports have demonstrated mechanisms of radioresistance, but the relationship of c-Myc and Fas in radiotherapy of lung adenocarcinoma remains unclear. In the present study, we established radiation-resistant A549 cell models (A549/R), and showed that there is an inverse expression pattern between c-Myc and Fas in A549/R cells. Notably, this study demonstrates for the first time that upregulation of Fas through inhibition of expression of c-Myc has an important role in irradiation-mediated lethality in A549/R cells. In addition, our results suggest that caspase-8-mediated Bid cleavage and the subsequent association with the mitochondrial pathway of apoptosis, which is primarily controlled by the Bcl-2 family proteins, are two critical events that are responsible for induction of apoptosis during irradiation-mediated cell death in A549 cells.

c-Myc is a common transcriptional factor. The amplification of c-Myc consequently results in an increased expression, which not only leads to the occurance of human cancers, but also influences the effect of cancer therapy (28). As a multifunctional protein, c-Myc has two distinct characteristics. One is the proto-oncoprotein property and the other is the tumor suppressor property, which leads to apoptosis, cellular senescence, and DNA damage responses through triggering the intrinsic barrier of cancer proliferation (29). As an oncogenic transcription factor, c-Myc can effectively master the balance point, as well as control accurately cell

proliferation and cell death. In this study, we found that the expression level of c-Myc was associated with Fas/FasL-meditated apoptosis. We also found that c-Myc affected the sensitivity of lung cancer cells to radiation, which was supported by previous studies (30-33), demonstrating that cancer radioresistance is also associated with the overexpression of c-Myc.

Fas death signaling pathway, as an important extrinsic apoptosis signaling pathways, is triggered by the interaction of FasL and Fas (34), which plays an important pro-apoptotic role in the apoptosis of receptor-mediated manner (35). Binding intimately with FasL, Fas is awakened to activate caspase-8, which forms the death inducing signaling complex (DISC). Caspase-8 oligomerizes its cleaved fragment release from the DISC to initiate the apoptotic program (36). Several lines of evidence showed that apoptosis is mediated by receptor or mitochondrial pathway leading to the successive activation of a series of caspases (37). Indeed, we found that FasL treatment induced the changes of pro-caspases, which further participated in c-Myc- and Fas-mediated lung cancer cell apoptosis.

Bcl-2 family is made up of anti- and pro-apoptotic members. The anti-apoptotic proteins include Bcl-2, Bcl-xL, Bcl-w and Mcl-1, and the pro-apoptotic family members contains Bax, Bak, Bid, Bad and Bim (38). Bid (a cytosolic, globular shaped protein) mainly consists of eight α helices and two central hydrophobic helices. Caspase-8 can cleave full length Bid into truncated fragment (tBid), which links caspase-8 activation together with mitochondrial death machinery to act as an intracellular bridge (39). tBid further actives Bax or Bak to increase the apoptotic activity (40,41). In our study we found upregulation of anti-apoptotic protein Bcl-2, while Bax was downregulated in c-Myc overexpression plasmid-treated cells compared with control treatment, supporting that Bcl-2 and Bax play important roles in c-Myc and Fas-mediated apoptosis.

In conclusion, this study proposed a novel relationship between c-Myc and Fas in A549 cell apoptosis induced by radiation. Ectopic expression of c-Myc substantially attenuates Fas-mediated lethality in A549 cells, whereas knockdown of c-Myc through siRNA significantly enhances Fas-mediated lethality in A549/R cells. The findings of this study will help in further understanding the molecular mechanism of radioresistance of lung carcinogenesis.

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