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Gamma Irradiation Upregulates B-cell Translocation Gene 2 to Attenuate Cell Proliferation of Lung Cancer Cells Through the JNK and NF-κB Pathways

Peihe Wang,* Yuanyuan Cai,* Dongju Lin,† and Yingxiao Jiang*

*Department of Radiotherapy, Weifang People's Hospital, Weifang, P.R. China
†Department of Reproduction, Weifang People's Hospital, Weifang, P.R. China

Gamma ray can promote cancer cell apoptosis and cell cycle arrest. It is often used in the clinical treatment of tumors, including lung cancer. In this study, we aimed to explore the role of gamma ray treatment and its correlation with BTG2 in cell proliferation, apoptosis, and cell cycle arrest regulation in a lung cancer cell line. A549 cell viability, apoptosis rate, and cell cycle were investigated after gamma ray treatment. We then used siRNA for BTG2 to detect the effect of BTG2 knockdown on the progress of gamma ray-treated lung cancer cells. Finally, we investigated the signaling pathway by which gamma ray might regulate BTG2. We found that gamma ray inhibited A549 cell viability and promoted apoptosis and cell cycle arrest, while BTG2 knockdown could relieve the effect caused by gamma ray on A549 cells. Moreover, we confirmed that the effect of BTG2 partly depends on p53 expression and gamma ray-promoting BTG2 expression through the JNK/NF-κB signaling pathway. Our study assessed the possible mechanism of gamma ray in tumor treatment and also investigated the role of BTG2 in gamma ray therapy. All these findings might give a deep understanding of the effect of gamma ray on the progression of lung cancer involving BTG2.

Key words: Lung neoplasms; Gamma ray; MAP kinase signaling system

INTRODUCTION

Gamma ray, also named low-linear energy transfer radiation, is one kind of strong penetrating power that can induce single-strand breaks (SSBs) and double-strand breaks (DSBs)[1,2]. Radiation therapy is used to treat multiple benign or malignant tumors, and about 50% of patients with cancer, including lung cancer, choose to receive radiotherapy[3–5]. Moreover, gamma ray has been widely reported to promote tumor cell apoptosis and cell cycle arrest and is used in the clinical treatment of tumors[6–8]. The study of Lacoste-Collin et al. has proven that a very small dose of gamma ray was able to modify the cellular response, including cell proliferation and apoptosis[9].

B-cell translocation gene 2 (BTG2) is one of the target genes of p53 in human cancers and is involved in a variety of biological processes including cell growth, development, differentiation, senescence, and death[10,11]. As a tumor suppressor gene that belongs to the antiproliferative gene family, BTG2 has been proven to mediate crosstalk between PI3K/Akt1 and NF-κB pathways, which regulates G_2/M arrest in both normal and cancer cells[12]. However, the molecular pathogenesis and the underlying mechanism of gamma ray treatment in lung cancer remain unclear.

In the present study, we aimed to explore the role of gamma ray treatment and its correlation with BTG2 in a lung cancer cell line. We detected the cell viability, cell apoptosis rate, and cell cycle arrest of A549 cells after gamma ray treatment and investigated the effects of gamma ray on BTG2 and p53 expression, as well as the regulation of the JNK/NF-κB signaling pathway. Our study explored the regulation mechanism of gamma ray in tumor treatment and also suggested a role for BTG2 in gamma ray therapy. All of our efforts might provide a theoretical basis and new insights about the role of gamma ray and BTG2 in the treatment of lung cancer.

MATERIALS AND METHODS

Cell Culture

The human A549 cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells incorporated with 4 mmol/L glutamine,

Address correspondence to Yingxiao Jiang, Department of Radiotherapy, Weifang People's Hospital, No. 151 Guangwen Street, Weifang 261000, P.R. China. E-mail: jiangyingxiao11@126.com

10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin were suspended in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Waltham, MA, USA). SP600125 (10 μM) and BMS-345541 (10 μM) were separately applied as JNK and NF-κB inhibitors in A549 cells.

Gamma Ray Treatment

The ^{60}Co theratron (Fyc-50H) machine was used to deliver the radiation (Irradiation Center, Faculty of Naval Medicine, Second Military Medical University, P.R. China). A549 cells were exposed to different doses of radiation (dose rate: 1 Gy/min) depending on the research requirement.

CCK-8 Assay

A549 cells were seeded into 96-well plates with 5×10^3 cells/well, and cell viability was assessed by cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Gaithersburg, MD, USA). Briefly, after stimulation the CCK-8 solution was added to the culture medium of each well and then incubated for 1 h at 37°C in humidified 95% air with 5% CO_2. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

Apoptosis Assay

Cell apoptosis analysis was performed using propidium iodide (PI)/fluorescein isothiocynate (FITC)-conjugated annexin V staining. Briefly, treated cells were washed with phosphate-buffered saline (PBS) and fixed in 70% ethanol. The cells were then washed twice with PBS and stained with PI/FITC–annexin V in the presence of 50 μg/ml RNase A (Sigma-Aldrich, St. Louis, MO, USA). Finally, they were incubated at room temperature for 1 h in the dark. Flow cytometry analysis was performed using a FACScan (Beckman Coulter, Fullerton, CA, USA).

Cell Cycle Assay

Briefly, cells were trypsinized after different treatments and washed twice in PBS, and then fixed overnight at −20°C in 300 μl of PBS and 700 μl of ethanol. The fixed cells were spun down gently in 200 μl of extraction buffer (0.1 % Triton X-100, 45 mM Na_2HPO_4, and 2.5 mM sodium citrate) at 37°C for 20 min and then stained with 50 μg/ml PI (BD Biosciences, San Jose, CA, USA) containing 50 μg/ml RNase A for 30 min at 37°C in the dark. Cell cycles were subsequently analyzed by FACS.

qRT-PCR

Total RNA was extracted with TRIzol reagent according to the manufacturer's protocol (Sigma-Aldrich), and 2 mg of RNA was reverse transcribed with the Omniscript RT kit (Qiagen, Milan, Italy) using random primers (1 mM) at 37°C for 1 h. Real-time (RT)-PCR was performed in 20-ml reaction volumes using the Power SYBER Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). All primers were purchased from Invitrogen (Carlsbad, CA, USA). RT-PCRs were carried out in a MJ Mini™ Personal Thermal Cycler apparatus (Bio-Rad Laboratories). Melting curves were obtained by increasing the temperature from 60°C to 95°C with a temperature transition rate of 0.5°C/s. We used comparative threshold cycle number (Ct) method to assess the relative quantification of gene expression. The fold change of the target gene was calculated as $2^{-\Delta\Delta}$Ct.

siRNA Transfection

Special siRNA for BTG2 or p53, and siRNA negative control (siNC) were designed and synthesized by GenePharma (Shanghai, P.R. China). Cell transfection was performed using Lipofectamine 3000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Western Blotting

Total cellular protein was extracted from A549 cells using RIPA lysis buffer (Beyotime, Shanghai, P.R. China). Proteins were quantified using a BCA Protein Assay Kit (Pierce, Bonn, Germany). Then samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Whatman, Dassel, Germany). The blots were blocked in 5% skim milk at room temperature for 1 h and incubated overnight with specific primary antibodies: Bax, Bcl-2, cleaved caspase 3, pro caspase 3, CDK2, cyclin E, p27, p21, c-Jun, p-c-Jun, p65, p-p65, IκB-α, p-IκB-α, BTG2, p53, and GAPDH (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C. Afterward, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 2 h. The bands were imaged using the WEST-ZOL-plus Western Blot Detection System.

Statistical Analysis

The results of multiple experiments are presented as the mean ± SD. Statistical analyses were performed using SPSS 19.0 statistical software. The p values were calculated using a one-way analysis of variance (ANOVA). A value of $p<0.05$ was considered to indicate a statistically significant result.

RESULTS

Gamma Ray Inhibited A549 Cell Viability and Promoted Cell Apoptosis and Cell Cycle Arrest

To better understand the effect mechanism of gamma ray on lung cancer cell progress, we exposed A549 cells

to different doses of radiation. In brief, we detected cell viability 0, 1, 2, and 3 days after cells were separately treated by gamma ray (0, 2, 4, and 8 Gy). The results suggest that gamma ray could inhibit cell viability (Fig. 1A). We then detected cell apoptosis by flow cytometry analysis and measured the expression of apoptosis-related factors by Western blot. The results suggest that gamma ray could promote cell apoptosis (Fig. 1B). We also investigated the effect of gamma ray on the cell cycle. We found that gamma ray induces A549 cell cycle arrest (Fig. 1C). Western blot assay results suggest that gamma ray treatment upregulated the apoptosis-related factors and downregulated antiapoptosis factors like Bcl-2, as well as downregulated the expressions of CDK2, cyclin E, promoting p27, and p21 (Fig. 2A and B).

Gamma Ray Promoted BTG2 Expression That Partly Depended on Increased p53 Expression

BTG2 is one of the p53 target genes and participates in cell proliferation and the cell cycle process. In the following research, we investigated the connection of gamma ray with BTG2 and p53. The results suggest that gamma ray promoted the expression of BTG2 and p53 (Fig. 3A and B). In A549 cells, siRNA inhibited expression of p53 (Fig. 3C). Moreover, the results showed that BTG2 was still upregulated even when p53 expression was inhibited by si-p53 transfection (Fig. 3D and E). This suggests that the effect of gamma ray on BTG2 partly relies on p53 expression.

BTG2 Knockdown Reduced Cell Damage, Cell Apoptosis, and Cell Cycle Arrest Caused by Gamma Ray

In the following experiment, we assessed the effects of BTG2 knockdown on cell viability, apoptosis, and cell cycle arrest caused by gamma ray. The results suggest that knockdown of BTG2 reduced the inhibition effect of gamma ray on A549 cell viability (Fig. 4A). We examined the effect of siRNA on protein expression of BTG2 (Fig. 4B). The results about the apoptosis rate and the expression of apoptosis-related factors suggest that knockdown of BTG2 inhibits the cell apoptosis, which was induced by gamma ray treatment (Fig. 4C). In the meantime, the expression of apoptosis-related factors was decreased, while Bcl-2 expression was increased (Fig. 4D). Finally, we monitored the cell cycle arrest after knockdown of BTG2. The results tell us that BTG2 inhibition alleviates the G_1/S cell cycle arrest caused by gamma ray treatment (Fig. 4E). Importantly, the expression of CDK2 and cyclin E was promoted, while the expression of p21 and p27 was inhibited (Fig. 4F).

Gamma Ray Promoted BTG2 Expression Through the JNK/NF-κB Signaling Pathway

Based on the results described above, we deeply researched the mechanism of gamma ray and BTG2

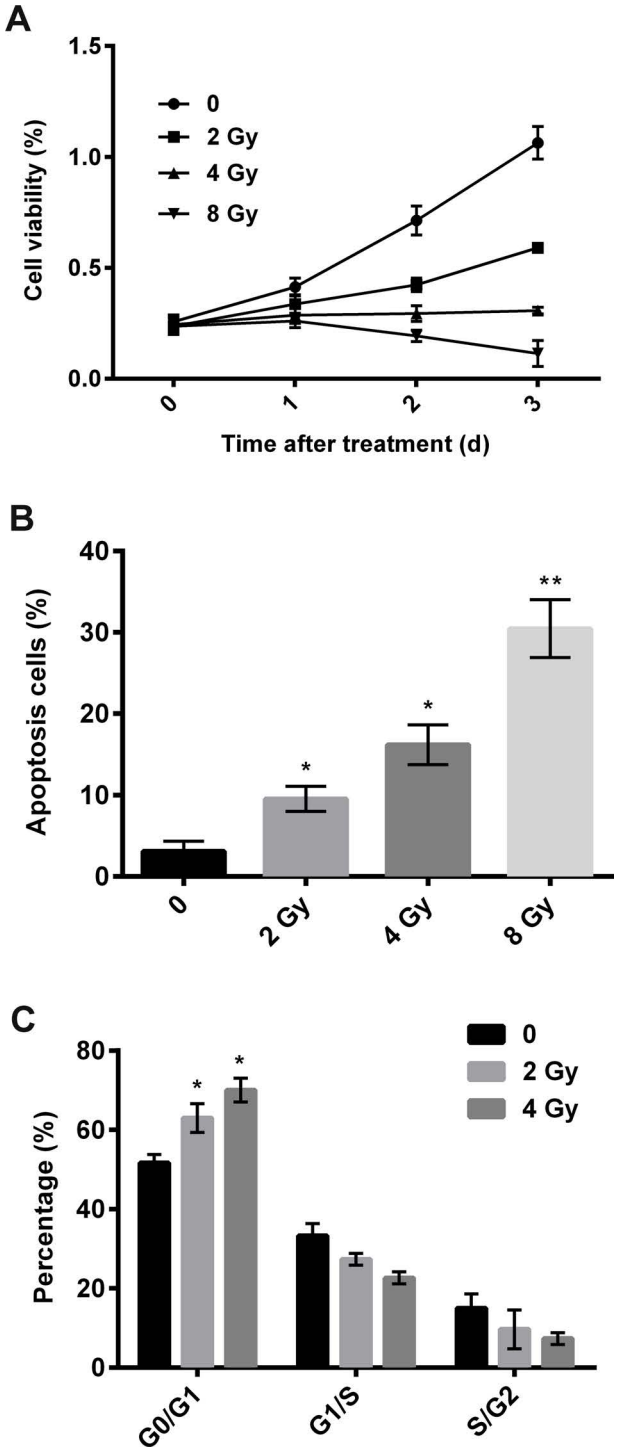

Figure 1. Effects of gamma ray on A549 cells. (A) Gamma ray inhibits cell viability. (B) Gamma ray promotes cell apoptosis. (C) Gamma ray promotes cell cycle arrest. *$p<0.05$, **$p<0.01$.

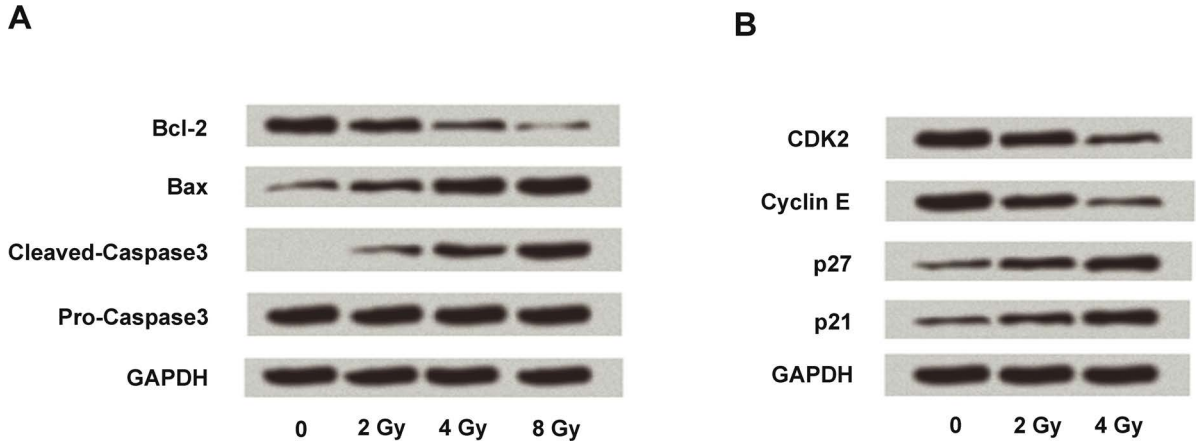

Figure 2. Effects of gamma ray on related protein expressions. (A) Gamma ray regulates expressions of apoptotic-related proteins. (B) Gamma ray regulated cell cycle factor expression.

regulation. We used SP600125 to inhibit the JNK signaling pathway and found that the NF-κB signaling pathway was inhibited. BTG2 was also downregulated (Fig. 5A). When the NF-κB signaling pathway was inhibited by BMS-345541, the JNK signaling pathway was not influenced, but BTG2 was downregulated (Fig. 5B). This suggested that NF-κB was the downstream of JNK signaling pathways, and gamma ray regulated BTG2 expression through the JNK/NF-κB signaling pathway.

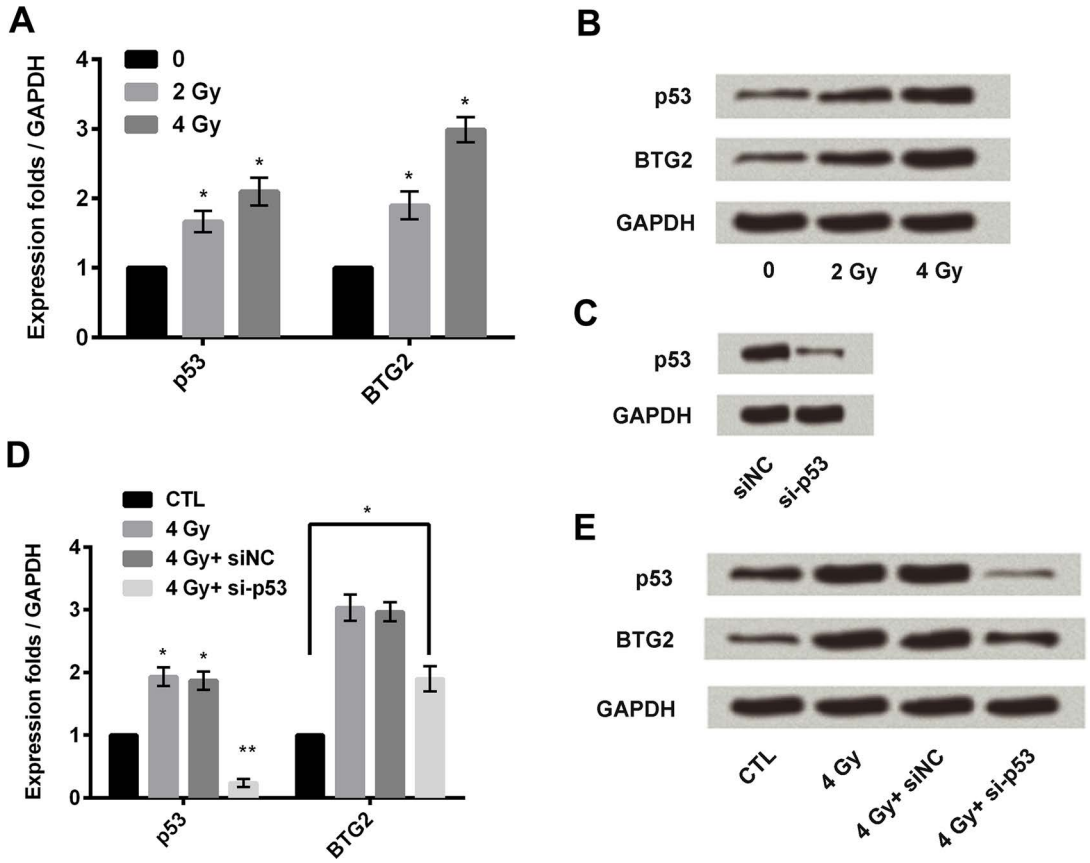

Figure 3. Connection of BTG2 and p53 expression. (A, B) Effects of gamma ray on BTG2 and p53 expression. (C) Effect of siRNA transfection on p53 expression in A549 cells. (D, E) Effects of p53 inhibition on BTG2 expression. *$p<0.05$.

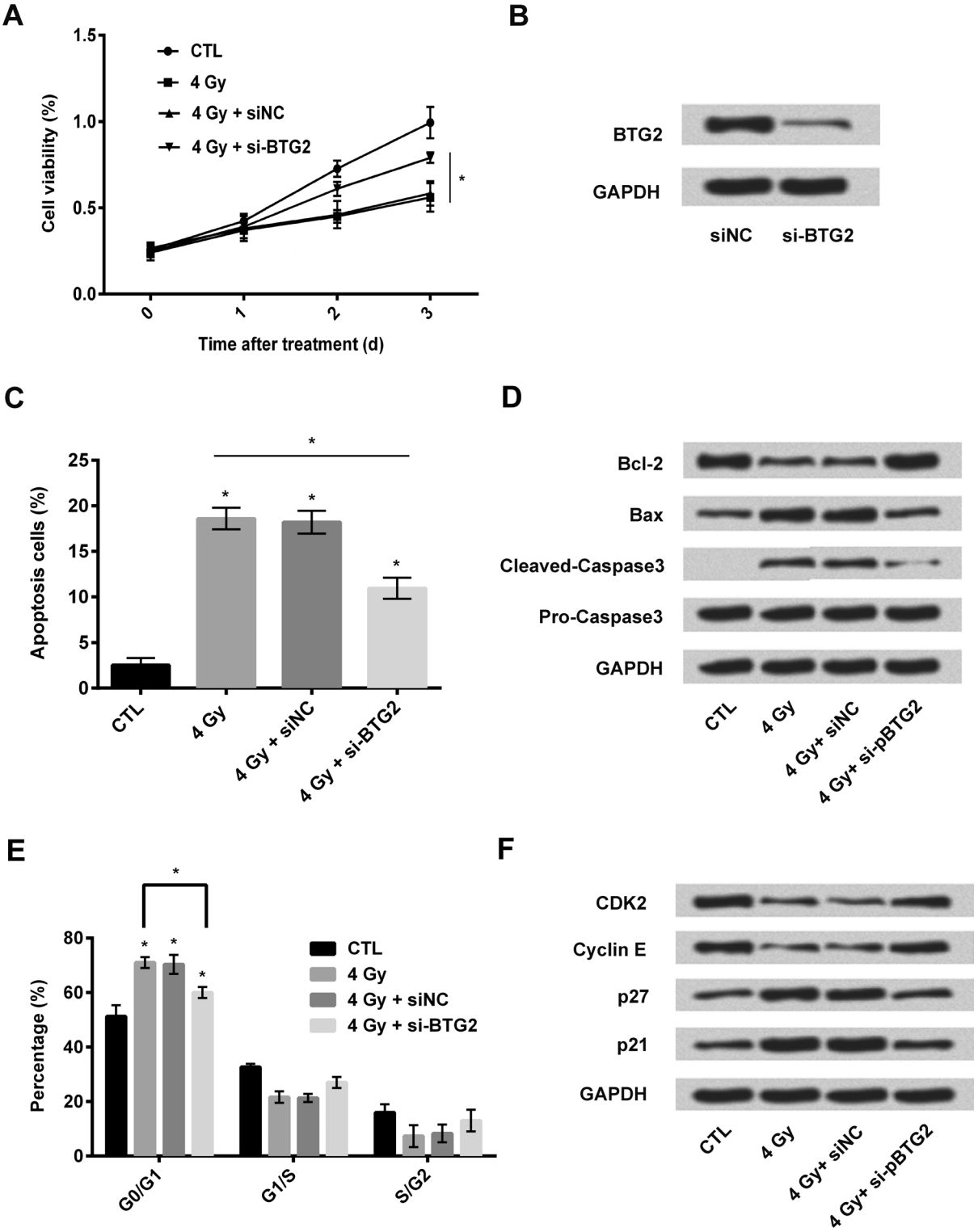

Figure 4. Effects of BTG2 knockdown on cell progress. (A) Effects of BTG2 knockdown on cell viability. (B) Effect of siRNA transfection on p53 expression in A549 cells. (C, D) Effects of BTG2 knockdown on cell apoptosis. (E, F) Effects of BTG2 knockdown on cell cycle arrest. *$p<0.05$.

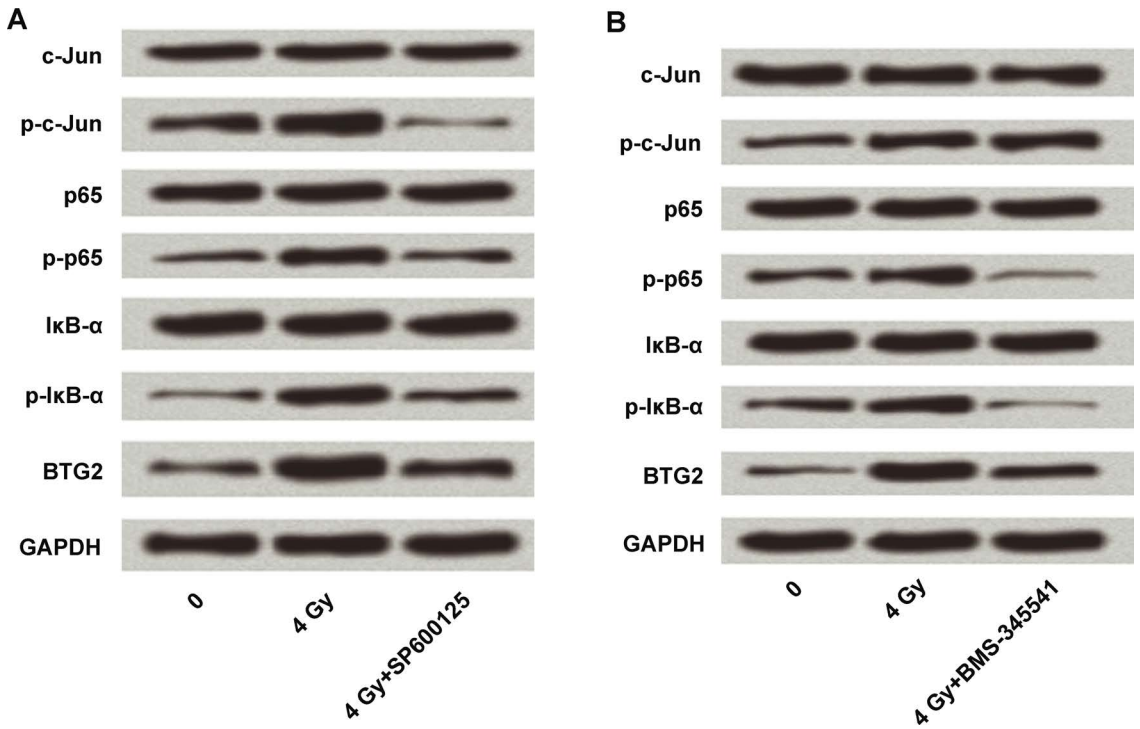

Figure 5. The regulation of gamma ray on BTG2 expression was related with JNK/NF-κB signaling pathway. (A) Effects of gamma ray combination with JNK inhibition on NF-κB and BTG2 expression. (B) Effects of gamma ray with NF-κB inhibition on JNK and BTG2 expression.

DISCUSSION

Lung cancer is the leading cause of cancer-related deaths worldwide and is still increasing in both morbidity and mortality[13,14]. Gamma ray is commonly used in the treatment of tumors, including lung cancer[15]. A deep understanding of the mechanism of how gamma ray regulates tumor cells can provide a theoretical basis for the development of new combined treatments that are of great importance. In the present study, A549 cells were purchased and exposed to different doses of radiation. We detected cell viability, apoptosis, and cell cycle. From the relative cell viability rate, we proved that gamma ray could inhibit A549 cell viability. Flow cytometry analysis and monitoring of cell apoptosis-related factor expression by Western blot suggested that gamma ray promoted cell apoptosis. In addition, the cell cycle assay results suggested that gamma ray induced A549 cell cycle arrest.

BTG2 is a tumor suppressor gene belonging to the antiproliferation gene family and has been reported to be downregulated in multiple human cancer cells[16–18]. It plays an important role in cell differentiation, proliferation, DNA damage repair, and apoptosis and is regulated by many factors in cancer cells[19–21]. Zhang et al. investigated the effects of BTG2 on the proliferation, apoptosis, and invasion of breast cancer cells and finally found that BTG2 inhibited cell proliferation and promoted apoptosis of breast cancer cells[22]. p53 is a transcription factor that plays a key role in tumor suppression and DNA damage, as well as controls the expression of proteins that are involved in cell apoptosis, cell cycle arrest, and DNA repair[23–26]. In our study, we detected BTG2 and p53 expression after gamma ray treatment, and the results show that gamma ray promoted the expression of BTG2 and p53. Moreover, knockdown of BTG2 reduced the cell damage, cell apoptosis, and cell cycle arrest caused by gamma ray.

Finally, we assessed the signaling pathway using signaling pathway inhibitors and found that gamma ray might promote BTG2 expression, cell apoptosis, and cell cycle arrest through the JNK/NF-κB signaling pathway. Taken together, these findings suggest that gamma ray might modulate BTG2 to attenuate cell proliferation of lung cancer cells through the JNK/NF-κB pathway. This might be helpful to obtain a deep understanding of the mechanism of gamma ray treatment in the progression of lung cancer and the role of BTG2 in gamma ray treatment.

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