

# Elevated BTG2 improves the radiosensitivity of non-small cell lung cancer (NSCLC) through apoptosis

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## Funding information

CAMS Innovation Fund for Medical Sciences, Grant/Award Number: 2021-I2M-1-042; National Natural Science Foundation of China, Grant/Award Number: 81730086

## Abstract

**Background:** To identify radio-responsive genes and explore the biological function of encoded proteins in non-small cell lung cancer (NSCLC).

**Methods:** Radio-responsive genes in irradiated H460 cells were screened from microarray data deposited in the Gene Expression Omnibus (GEO) database. A quantitative real time polymerase chain reaction assay was used to detect the expression of candidate radio-responsive genes in irradiated cells. CCK-8 assay, EDU assay, clone formation assay, immunofluorescence and flow cytometry were conducted to evaluate the biological function of B cell translocation gene 2 (BTG2) in NSCLC.

**Results:** Bioinformatic analysis using GES20549 showed that BTG2 was a radio-responsive gene in irradiated H460 cells. The mRNA expression level of BTG2 was lower in H460 cells compared with that in BEAS-2B normal lung epithelial cells. BTG2 expression was elevated upon IR exposure, in a dose-dependent but not a time-dependent manner. CCK-8 and EDU assays revealed that BTG2 overexpression inhibited the growth rate of irradiated cells. Clone formation showed that elevated BTG2 promoted DNA damage of irradiated H460 cells. The number of  $\gamma$ -H2AX foci induced by DNA damage was also markedly increased upon BTG2 overexpression. Flow cytometry showed that BTG2 increased IR-induced cell apoptosis.

**Conclusions:** BTG2 may be a novel radio-responsive factor and a promising therapeutic target for radiotherapy of NSCLC.

## KEYWORDS

bioinformatics, biological function, BTG2, H460 cells, radio-responsive genes

## INTRODUCTION

Lung cancer is the second most common diagnosed cancer worldwide, and mostly accounts for high morbidity and mortality rates.<sup>1</sup> Lung cancer is divided into two types, small cell lung cancer and non-small cell lung cancer (NSCLC), and NSCLC constitutes approximately 85% of all lung cancer cases.<sup>2</sup> Surgical resection is currently considered the best treatment strategy for early-stage NSCLC.<sup>3</sup> For inoperable patients or patients who refuse surgery, radiation therapy is extensively applied.<sup>3,4</sup> However, radio-resistance to ionizing radiation (IR) is a major obstacle for the successful treatment of NSCLC as it can result in local recurrence and distant metastasis in the clinic, and greatly limits the

application of radiation therapy.<sup>5</sup> Therefore, searching for the mechanisms underlying radio-resistance in NSCLC is a critical research focus.

Several studies have shown that various factors affect the response of NSCLC to IR, including endogenous RNAs molecules (mRNA, miRNA and lncRNA) and exogenous molecules (small molecule inhibitors).<sup>6–10</sup> IR also leads to a variety of changes in cancer, especially at the transcription level, providing the new thoughts for the study of molecular mechanism.<sup>11,12</sup> For example, Yang et al. found that IR reduced COMMD10 expression, which increased the radio-resistance of hepatocellular carcinoma through inhibiting the HIF1 $\alpha$ /CP loop.<sup>13</sup> Free open access data sources are an invaluable tool research, as they provide a massive amount

of genetic information at the transcriptional and post-transcriptional levels.<sup>14</sup> The Gene Expression Omnibus (GEO) database is one of the most accessible and well-established data sources and supported by the National Center for Biotechnology Information. The GEO provides a large amount of microarray data for genetic research. Analysis of the high-throughput data from the GEO database has been broadly applied in cancer research.<sup>16,17</sup>

In this study, we aimed to identify potential radio-responsive genes in NSCLC through bioinformatic analysis using the GEO database. We evaluated gene expression changes in NSCLC cells after IR treatment and explored the potential biological mechanism of the identified candidates in irradiated cells.

## METHODS

### Data acquisition and analysis

Gene expression data from H460 cells treated with 2 Gy were obtained from the GSE20549 dataset in the GEO database. Raw data were analyzed by normalizing BetweenArrays function in the limma package in R software (Version 4.1.2). The differentially expressed genes (DEGs) in irradiated H460 cells at different time points were separately extracted from the processed gene expression data through the limma package. The heatmap was created using the pheatmap package in R based on DEGs. The adjusted  $p$ -value  $< 0.05$  and  $|\log_2FC$  (fold change)|  $\geq 1$  were selected as the cutoff criteria.

### Cell lines and cell culture

NSCLC H460 cells and BEAS-2B human normal lung epithelial cells were purchased from the American-Type Culture Collection. The cells were cultured in RPMI-1640 medium (HyClone) supplemented with 10% fetal bovine serum (HyClone), and 1% penicillin–streptomycin solution (Solarbio) in 5% CO<sub>2</sub> at 37°C.

### Cell transfection and radiation treatment

Cells were seeded in 6-, 24- or 96-well plates until they were approximately 70%–80% confluent. Transfection was performed using Lipofectamine 3000 (Invitrogen) in accordance with the manufacturer's instructions. At 24 h after cell transfection, H460 cells were exposed to 2 Gy gamma radiation generated from a <sup>137</sup>Cs-ray source at a dose rate of 0.99 Gy/min.

### Plasmid construction

The coding sequence of the human BTG2 gene was cloned into the pEGFP-C1 vector (Clontech). shRNA sequences

targeting BTG2 were cloned into the pRNAT-U6.1/Neo vector (Clontech). The shRNA sequences are as follows: shBTG2-1 5'-GAAACAAAGCTGTGAATCATTCAAGA GATGATTCACAGCTTTGTTTCTTTTTT-3' and shBTG2-2 5'-GCATTCGCATCAACCACAAGATTCAAGAGATC TTGTG GTTGATGCGAATGCTTTTTT-3'.

### qRT-PCR

Total RNA was obtained from cells using Trizol (Takara) following the manufacturer's protocol. cDNA was synthesized using a reverse transcription kit (Japan). qRT-PCR was performed with cDNA as template using the 2X SYBR Green Master Mix (Thermo). The forward primer of GAPDH was 5'-ATCACCATCTTCC AGGAGCGA-3' and reverse was 5'-CCTTCTCCATGGTGGTGAAGAC-3'; The forward primer of BTG2 was 5'- GCACTCACAGAGCACTACAA-3' and reverse was 5'- TGCGGTAGGACACCTCATA-3'. Relative expression level of BTG2 was normalized to GAPDH.

### Western blot

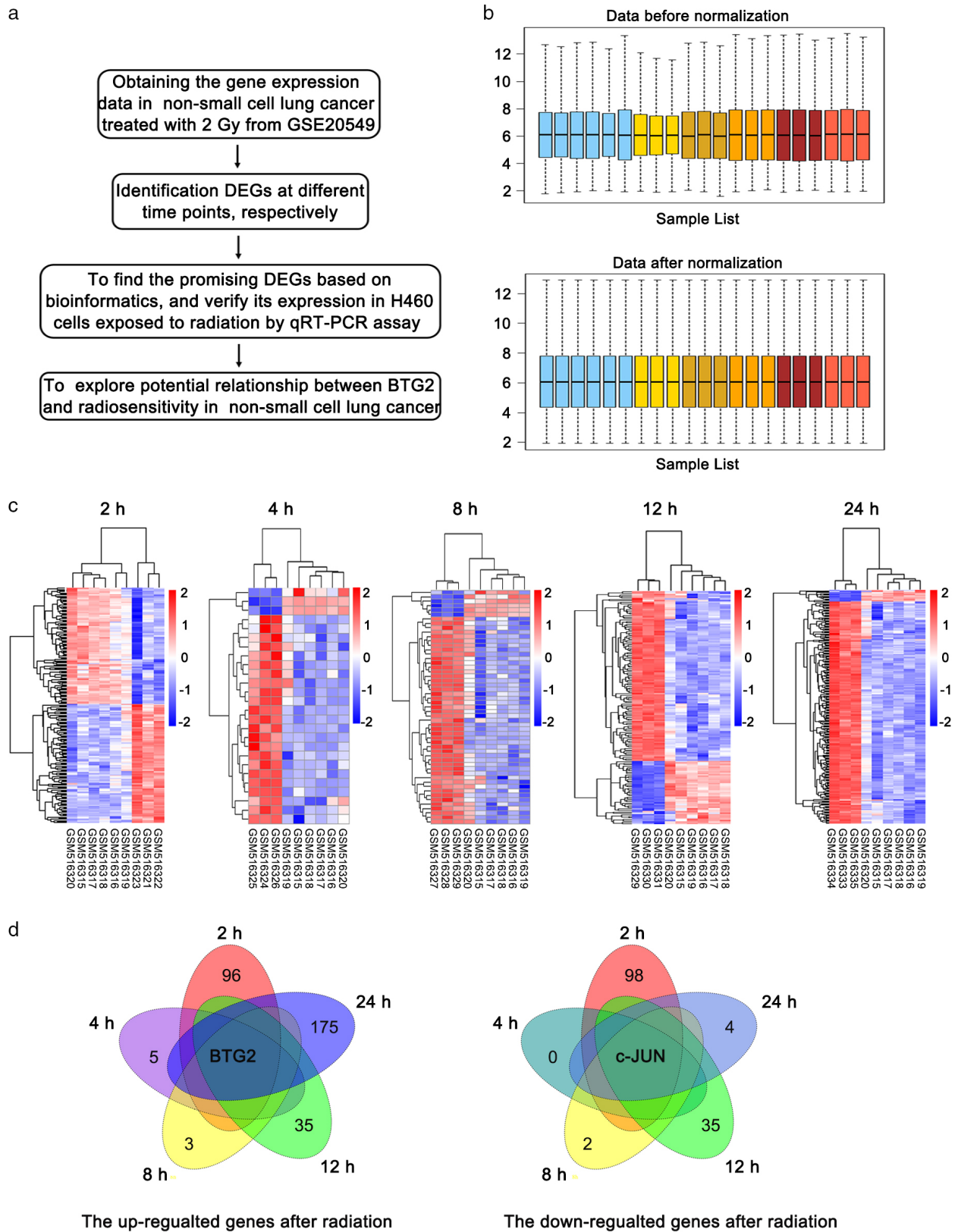
Cellular proteins were extracted from H460 cells using RIPA buffer (Solarbio), separated on a 15% SDS PAGE gel and then transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 5% nonfat dry milk in TBST for 1 h at room temperature. Next, the membranes were incubated with anti-BTG2 (diluted 1:100, Santa Cruz) and anti-GAPDH (diluted 1:5000, Proteintech) overnight at 4°C. The membrane was then incubated with Rabbit IgG HRP-conjugated Antibody (diluted 1:5000, Proteintech) diluted in TBST at 37°C for 40 min. Protein signals were detected using the eECL Western Blot Kit (CWBio).

### CCK-8 assay

Cell proliferation was evaluated using cell counting kit-8 (CCK-8) (Solarbio) in accordance with the manufacturer's instructions. Cells (2000) were seeded into 96-well plates and at specific time point, the absorbance at 490 nm (OD value) was measured using an auto-microplate reader. Each experimental group was tested in six replicated wells.

### EDU assay

The cells were seeded in 24-well plates. After they achieved 70% confluence, transfection was performed. Cells were then exposed to 2 Gy gamma radiation and cultured for 24 h. Cell growth was detected using an EdU Cell Proliferation Assay Kit (Ribobio) in accordance with the manufacturer's instructions.



**FIGURE 1** Identification of the radio-responsive genes in irradiated H460 cells using GSE20549. (a) A flow chart for the experimental design. (b) Normalization of raw data. (c) Cluster analysis of raw data. (d) Identification of the outstanding radio-responsive genes by overlapping from up- and down-regulated DEGs

## Clone formation

Cells were seeded in 6-well plates, transfected, and then exposed to IR after 24 h. The cells were then cultured for approximately 2 weeks until colonies formed. The plate was washed with water and cells were stained with crystal violet. The number of colonies was counted using ImageJ software.

## Immunofluorescence assay

Cells cultured on coverslips were fixed in 4% paraformaldehyde for 30 min and permeabilized using 0.5% Triton X-100 for 15 min, followed by incubation in 1% BSA solution for 2 h. The slides were stained overnight at 4°C with phosphohistone H2A.X (Ser139) (20E3) Rabbit mAb (diluted 1:100, Cell Signaling Technology) and then incubated with Goat Anti-Rabbit IgG H&L (Alexa Fluor 488) (diluted 1:2000, Abcam) and 10 µg/ml DAPI solution for 2 h at room temperature. The coverslips were mounted on glass slides with fluorescent mounting media. Cells were visualized using a confocal microscope (LSM880 AxioObserver).

## Statistical analysis

All data were analyzed using SPSS 22.0 and statistical significance was assessed by comparing mean values ( $\pm$ SD) using a Student's *t*-test. All experiments were performed at least three times. *p*-values < 0.05 was considered statistically significant.

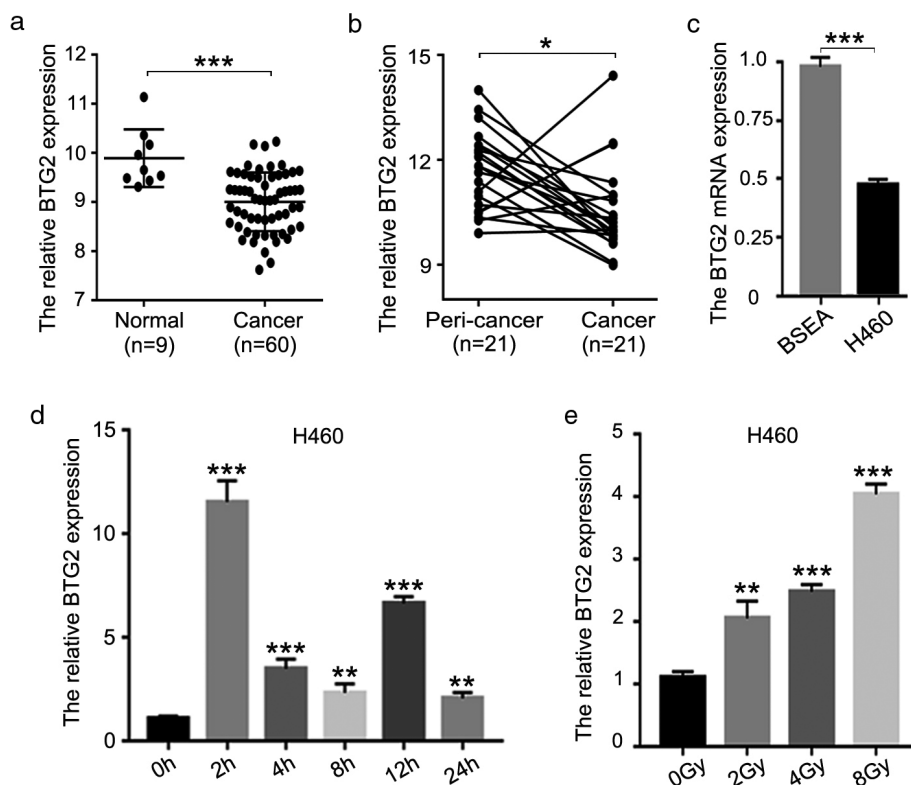
## RESULTS

### BTG2 is a potential radio-responsive gene in H460 cells

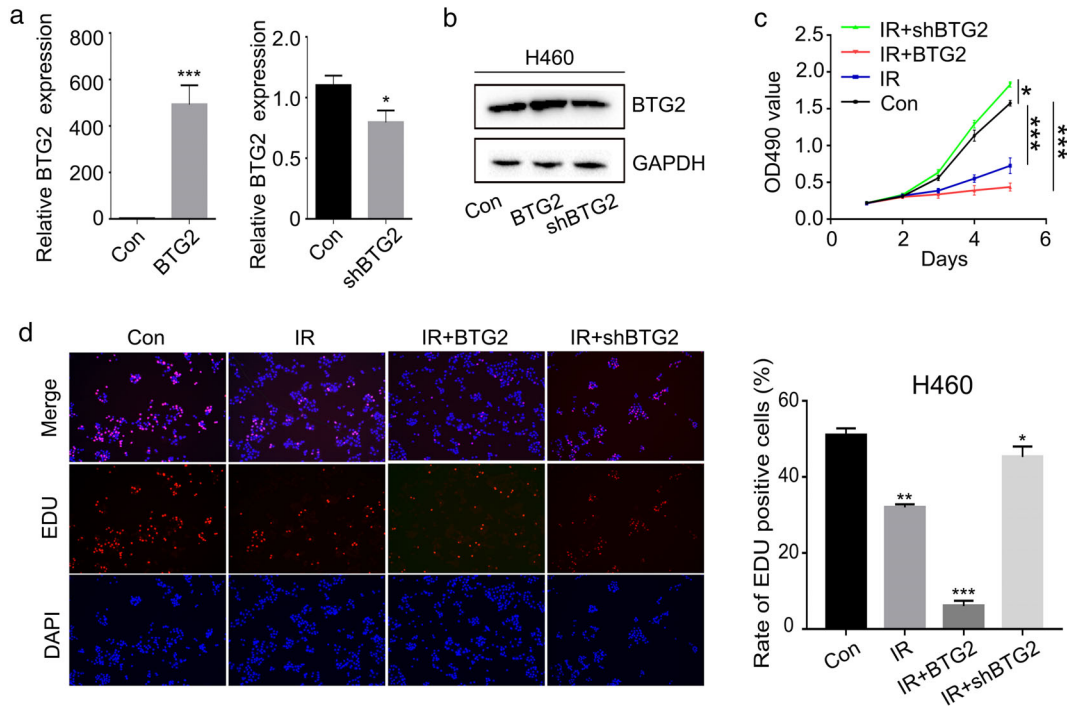
To identify radio-responsive genes in NSCLC, we examined the time-series gene expression profiles in H460 cells using data from GSE20549, as shown in Figure 1a. The raw data from GSE20549 were normalized to eliminate the non-experimental error (Figure 1b). The results of cluster analysis showed that DEGs at different time points could be clearly identified and distinguished (Figure 1c). Using the selection criteria defined in the Methods, we identified DEGs in irradiated cells at different time points. The DEGs are shown in Figure S1. Notably, the BTG2 gene was upregulated at all time points, and the c-JUN gene was downregulated at all time points (Figure 4d). These results suggest that BTG2 and c-JUN may be potential radio-responsive genes in H460 cells.

### IR upregulated BTG2 expression in H460 cells in a dose-dependent manner

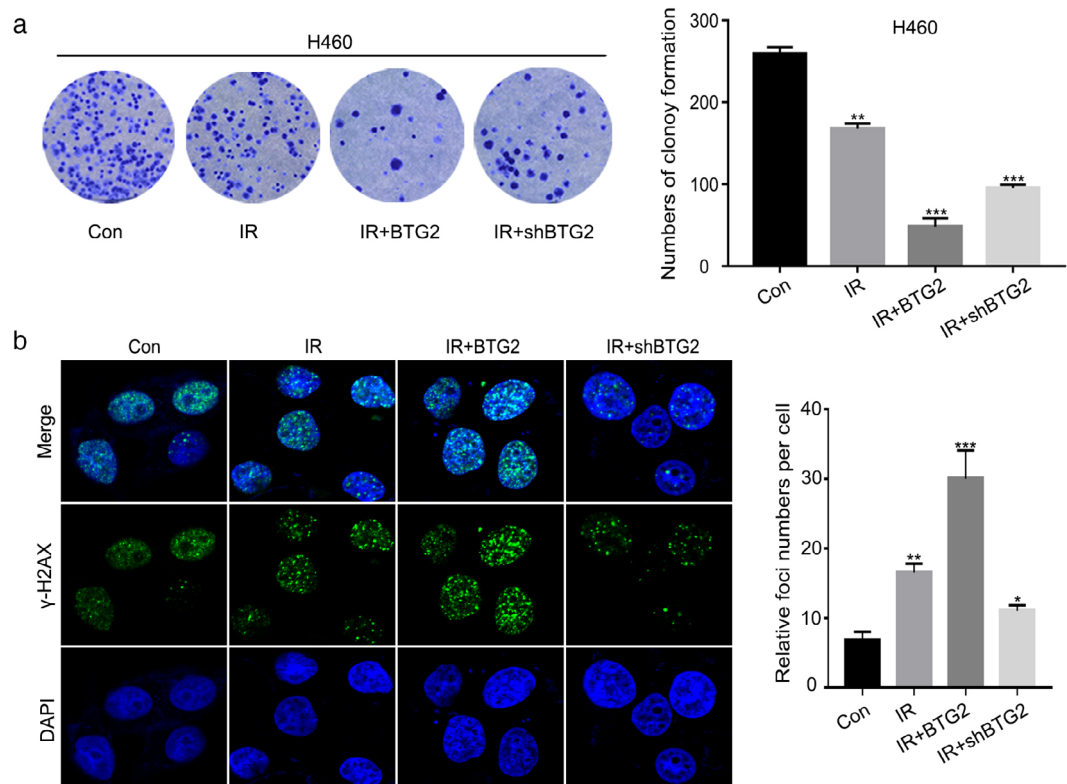
We next examined BTG2 expression in NSCLC tissues using data from GEO datasets. The results showed that BTG2 expression was significantly downregulated in NSCLC samples compared with no-tumor lung samples (Figures 2a,b). qRT-PCR also showed that the mRNA expression level of BTG2 was lower in H460 cells than in BEAS-2B human normal lung epithelial cells (Figure 2c). These results suggest



**FIGURE 2** The expression level of BTG2. (a) The relative BTG2 expression in NSCLC and nontumor lung tissues in GSE103512. (b) The relative BTG2 expression in NSCLC and tumor adjacent tissues in GSE21933. (c) The relative BTG2 mRNA expression in NSCLC and BEAS-2B cells. (d) The relative BTG2 mRNA expression in irradiated H460 cells at different time points. (e) The relative BTG2 mRNA expression in H460 cells irradiated at different doses

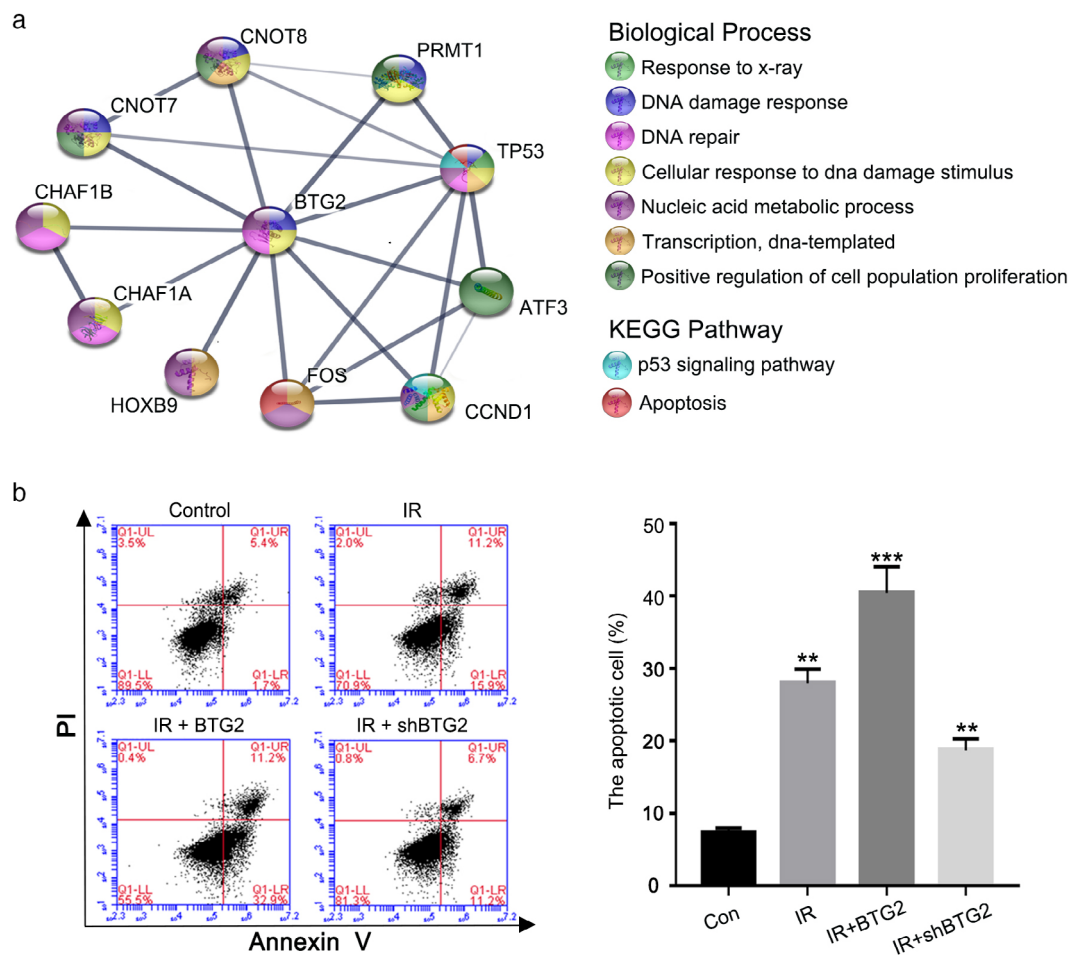


**FIGURE 3** BTG2 restrained cell growth of H460 cells treated with IR. (a) The relative mRNA expression level of BTG2 in H460 cells after transfection. (b) The relative protein expression level of BTG2 in H460 cells after transfection. (c) CCK-8 assay of cell growth rate in cells treated as indicated at different time points after IR exposure. (d) EDU assay of cell growth rate in cells treated as indicated at 24 h after IR exposure



**FIGURE 4** BTG2 increases IR-induced DNA damage. (a) Colony formation assays of irradiated H460 cells. (b) Immunofluorescence of  $\gamma$ -H2AX foci in irradiated H460 cells





**FIGURE 5** BTG2 increases IR-induced cell apoptosis. (a) The main biological processes and pathways involved in BTG2 were explored using the STRING database. (b) Flow cytometry showed the effect of BTG2 on cell apoptosis in irradiated H460 cells

that BTG2 may be a tumor suppressor in NSCLC. To verify the accuracy of high-throughput data, H460 cells were selected as the primary experiment cell line in this study, as this cell line is commonly used in NSCLC research. We evaluated BTG2 expression in H460 cells after IR treatment. BTG2 expression level showed an increasing trend after IR exposure (Figures 2d,e). In addition, BTG2 expression exhibited an increasing trend in a dose-dependent but not in a time-dependent manner. Notably, qRT-PCR assay showed that IR increased c-JUN mRNA expression, which is inconsistent with the results of bioinformatic analysis (Appendix S1). These findings suggest that BTG2 might be a radio-responsive factor in NSCLC.

### BTG2 restrained the cell growth of H460 cells treated with IR

We next examined the cell growth rate of irradiated H460 cells transfected with a BTG2 overexpression plasmid or plasmid expressing shRNA targeting BTG2. Up- or down-regulated BTG2 expression was verified by qPT-PCR and western blot assay (Figure 3a,b). CCK-8 assay showed that

while IR significantly repressed the cell growth of H460 cells, BTG2 overexpression further decreased cell growth and BTG2 knockdown increased growth (Figure 3c). EDU assay also demonstrated that BTG2 overexpression reduced the cell growth of irradiated H460 cells (Figure 3d). This effect of inhibition was statistically significant before and after transfection treatment. Together, these data suggested that BTG2 has a repressive effect on the cell growth of irradiated H460 cells.

### BTG2 enhanced the IR-induced DNA damage of H460 cells

We next explored the mechanism of BTG2 in H460 cells under IR stress and hypothesized that BTG2 may influence DNA damage in response to IR. We found that H460 cells transfected with pEGFP-C1/BTG2 exhibited a stronger effect on IR-induced DNA damage using clone formation (Figure 4a). The number of  $\gamma$ -H2AX foci reflects the degree of DNA damage. We evaluated the number of  $\gamma$ -H2AX foci at 24 h after IR by immunofluorescence assay. IR combined with BTG2 expression showed a dramatic increase in

$\gamma$ -H2AX foci formation compared with the IR group (Figure 4b). These data suggested that BTG2 enhanced the IR-induced DNA damage of H460 cells.

## BTG2 enhanced the IR-induced apoptosis of H460 cells

To evaluate the function of BTG2 in NSCLC, we established the PPI network involving BTG2 using the STRING database. The results showed that there were 11 nodes and 20 interactions in the PPI network (Figure 5a). GO enrichment analysis showed that the BTG2-associated biological processes were markedly enriched in DNA damage response, DNA repair, and cellular response to DNA damage stimulus. KEGG pathway analysis revealed that apoptosis-associated signaling pathways, such as the p53 signaling pathway, were associated with BTG2. We then performed flow cytometry to examine the effects of BTG2 on the cell apoptosis after 24 h under IR exposure. The results showed that BTG2 overexpression increased IR-induced cell apoptosis (Figure 5b). Together, these results implied that BTG2 plays a role in irradiated H460 cells that is closely associated with cell apoptosis.

## DISCUSSION

Radiation therapy has been a systematic treatment modality of NSCLC for decades, but radio-resistance has an adverse impact on the survival of many patients against cancer.<sup>18</sup> Therefore, better understanding of the mechanisms underlying radio-resistance in NSCLC is critical. Recent studies have shown that RNA molecules play an important role on radio-resistance, especially mRNAs.<sup>19,20</sup> Therefore, identifying radio-responsive genes and the biological function of encoded proteins in NSCLC may lead to the establishment of novel therapeutic targets to enhance the efficacy of radiation therapy and improve the prognosis of patients.

High-throughput data generated from next-generation sequencing technology has been extensively used in cancer research and are available in various public databases.<sup>21,22</sup> The GEO database is an open-access database with a large amount of transcript information, especially cancer-associated transcripts, from different species.<sup>15</sup> The use of GEO databases in research has grown with the development of bioinformatics.<sup>23</sup> For example, Ma et al. identified that the CENPK gene might be a potential new gene for diagnosis and prognosis in lung cancer using data from the GEO database.<sup>24</sup> In vitro experiments showed that CENPK might play an important role in the progression of lung adenocarcinoma. Therefore, the combination of microarray data in GEO database combined with biological experiments may lead to better understanding of the mechanisms of the development of lung cancer.

In this study, we obtained the raw data from GSE20549 to identify potential radio-responsive genes in NSCLC and explore their biological functions in irradiated cells. Our

results identified two candidate genes with altered expression levels after IR treatment, the BTG2 and c-JUN genes. In detail, BTG2 expression was increased at different time points after IR treatment. By contrast, c-JUN expression was markedly decreased upon IR stress. However, the expression of c-JUN in vitro results was inconsistent with bioinformatic results. Therefore, we pursued BTG2 as a potential radio-responsive factor.

The B cell translocation gene 2 (BTG2) is one of the BTG/TOB family genes. BTG2 is expressed at low levels in multiple cancers and might therefore act as a tumor suppressor.<sup>25–27</sup> BTG2 plays an important role in cell proliferation, migration, invasion and drug resistance.<sup>28,29</sup> Little research has been performed on the biological behavior of BTG2 in response to IR stress. Guo et al. reported that miRNA-29b regulates the radiosensitivity of esophageal squamous cell cancer by targeting BTG2.<sup>30</sup> However, the relationship between BTG2 expression and IR in lung cancer has not yet been fully determined. Among NSCLC cells, H460 cells are commonly used in research studies. To verify the accuracy of high-throughput data, H460 cells were selected as the primary experiment cell line in this study. First, we evaluated the mRNA expression of BTG2 in H460 cells treated with 2Gy by qRT-PCR assay and found that BTG2 mRNA expression was elevated in IR-hit cells in a dose-dependent manner. This result was in line with the bioinformatic findings. These data indicate that the BTG2 gene was a radio-responsive gene, which might exhibit a stronger sensitivity to IR in NSCLC. We then examined the biological functions of BTG2 in irradiated cells by modulating BTG2 expression in cells. CCK-8 and EDU assays showed that BTG2 had a markedly inhibitory effect on the cell growth of irradiated cells. DNA damage is a common phenotype of tumor cells exposed to IR. Clone formation showed that BTG2 expression enhanced the DNA damage in irradiated cells. The formation of  $\gamma$ -H2AX foci is another indicator of DNA damage. We found that the number of  $\gamma$ -H2AX foci was markedly increased in irradiated cells upon BTG2 overexpression. STRING database analysis showed that BTG2 might function in cell apoptosis and flow cytometry indicated that BTG2 enhanced IR-induced cell apoptosis. These findings suggest that BTG2 might be involved in apoptosis-associated signaling pathways, and this will be explored in further studies.

In summary, this study demonstrates that BTG2 is expressed in response to IR in NSCLC and reduces cell growth and increases apoptosis. The specific mechanism of BTG2 in the IR response in NSCLC requires further exploration in vitro and in vivo.

## ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (No.81730086) and, the CAMS Innovation Fund for Medical Sciences (No. 2021-I2M-1-042). We thank Gabrielle White Wolf, from Liwen Bianji (Edanz) ([www.liwenbianji.cn](http://www.liwenbianji.cn)) for editing the English text of a draft of this manuscript.

**CONFLICT OF INTEREST**

The authors do not report any conflict of interest.

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**SUPPORTING INFORMATION**

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**How to cite this article:** Zhu C, Zhang S, Xue A, Feng G, Fan S. Elevated BTG2 improves the radiosensitivity of non-small cell lung cancer (NSCLC) through apoptosis. *Thorac Cancer*. 2022;13: 1441–8. <https://doi.org/10.1111/1759-7714.14410>