




# Increased activation of cGAS-STING pathway enhances radiosensitivity of non-small cell lung cancer cells

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## Abstract

**Background:** Radiotherapy is an effective therapeutic approach widely used clinically in non-small cell lung cancer (NSCLC), but radioresistance remains a major challenge. New and effective radiosensitizing approaches are thus urgently needed. The activation of DNA-sensing cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway has become an attractive therapeutic target, but the relationship between activation of cGAS-STING pathway and radiosensitization of NSCLC cells remains unknown.

**Methods:** Considering low expression of cGAS-STING pathway genes in NSCLC, including STING, we used an activator (STING agonist, dimeric amidobenzimidazole [diABZI]) of cGAS-STING pathway and increased activation factor (DNA double strand breaks) of cGAS-STING pathway to respectively reinforce the activation of cGAS-STING pathway in NSCLC cells. We then investigated the effect of increased activation of cGAS-STING pathway on the proliferation of H460 and A549 cells by CCK-8 and colony formation assays, and revealed the underlying mechanism.

**Results:** We found that both diABZI and the increased DNA double strand breaks could sensitize NSCLC cells to irradiation. Mechanically, our results showed that the increased activation of cGAS-STING pathway enhanced radiosensitivity by promoting apoptosis in NSCLC cells.

**Conclusion:** Taken together, we concluded that diABZI could be used as a radiosensitizer in NSCLC cells, and targeting the activation of cGAS-STING pathway has a potential to be a new approach for NSCLC radiosensitizing.

## KEYWORDS

apoptosis, cGAS-STING pathway, diABZI, non-small cell lung cancer, radiosensitivity

## INTRODUCTION

Lung cancer is one of the most commonly diagnosed cancers and remains the main cause of cancer-related death worldwide.<sup>1</sup> Among the common types of lung cancer, ~85% of clinical cases are non-small cell lung cancer (NSCLC),<sup>2,3</sup> which has low overall cure and survival rates.<sup>4,5</sup> Radiotherapy plays a prominent role in the treatment of NSCLC,<sup>6</sup> but the intrinsic or acquired radioresistance greatly impairs the efficacy of radiotherapy,<sup>7</sup> resulting in its failure. Hence, novel and effective radiosensitizing methods are

urgently needed to improve the efficacy of radiotherapy for NSCLC and enhance the survival rate in patients.

Recently, the cyclic GMP-AMP (cGAMP) synthase (cGAS)-stimulator of interferon genes (STING) pathway has gained enormous attention due to its close relationship with antitumor immunity, cancer progression, tumor microenvironment and cancer biotherapy.<sup>8,9</sup> cGAS, the cytosolic DNA sensor, can recognize free DNA, and their binding triggers the production of cGAMP, which stimulates STING. TANK binding kinase 1 (TBK1) is then recruited to form STING-TBK1 complex. After that, the signal cascades and

culminates in the nucleus, mediated by interferon regulatory factor 3 (IRF3) and NF- $\kappa$ B, leading to interferon (IFN) secretion.<sup>8,10</sup> In radiotherapy, the produced DNA damage can induce the formation of free DNA in the cytoplasm, activating the DNA-sensing cGAS-STING pathway.<sup>8,11</sup> Moreover, this pathway activation has the potential to improve the efficacy of immune checkpoint inhibitors and to stimulate anticancer immune response,<sup>8–10</sup> leading to the activation of cGAS-STING pathway becoming an attractive therapeutic target. Whether the regulation of cGAS-STING pathway activation could lead to changes in the radiosensitivity of NSCLC, or whether increased activation of cGAS-STING pathway could sensitize NSCLC cells to irradiation (IR) remains to be elucidated.

In radiotherapy, the produced DNA double strand breaks (DSBs) have been found to activate the DNA-sensing cGAS-STING pathway,<sup>8,11,12</sup> so increasing DSBs by an inhibitor of DNA repair is an available method to reinforce the activation of cGAS-STING pathway. On the other hand, in the cGAS-STING pathway, STING is a promising molecular target with its essential role in regulating the transcription of plentiful host defense genes (including type I IFN) and protecting cells against cancer development.<sup>13,14</sup> It is therefore speculated that increased activation of the cGAS-STING pathway through STING activation is also available to study the role of cGAS-STING pathway activation in the radiosensitization of NSCLC. A newly developed small molecule dimeric amidobenzimidazole (diABZI), a STING agonist, has been reported to elicit strong antitumor activity in immunocompetent mice with established syngeneic colon tumors.<sup>15</sup> Pharmacological activation of STING by diABZI has also been found to sensitize melanoma cells to BRAF inhibitors, potentiating cell death.<sup>16</sup> Additionally, diABZI could potentially inhibit SARS-CoV-2 infection by transiently stimulating IFN signaling.<sup>17</sup> So far, it is unknown whether diABZI sensitizes NSCLC cells to IR.

Here, we used the small molecule diABZI (an activator of cGAS-STING pathway) and increased DSBs (activation factor of cGAS-STING pathway) to respectively examine the role of cGAS-STING pathway activation in the radiosensitization of NSCLC cells. Our data showed that the reinforced activation of cGAS-STING pathway could effectively enhance the radiosensitivity of NSCLC cells.

## METHODS

### Cell culture

Human NSCLC cell lines (H460 and A549) were cultured in RPMI 1640 (Gibco) and Ham's F-12 K (Procell), respectively. The human bronchus epithelial cell line BEAS-2B (normal cell) was cultured in DMEM (Gibco). Meanwhile, 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin solution were added into all the cell culture media. Cells were maintained in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

### Irradiation studies

Irradiation (IR) was carried out with a <sup>137</sup>Cs-irradiator (Gammacell-40, Atomic Energy of Canadian Inc.) at a dose rate of 0.9 Gy/min. Cells were irradiated with 0, 2 and 4 Gy  $\gamma$ -ray after pretreatment with diABZI STING agonist-1 trihydrochloride (MedChemExpress, cat no. HY-112921B) or DMSO for 2 h. In this study, diABZI was used at a 20 nM concentration according to previous studies.<sup>15,16</sup>

### CCK-8 assay

Briefly, cells ( $3 \times 10^3$  cells/well) were plated in 96-well plates and when they had grown to 70%–80% confluence, were treated with 20 nM diABZI or DMSO. After 24, 48 and 72 h treatment, respectively, cell counting kit-8 (CCK-8) (Biosharp) was added to each well. After incubation for 3 h, absorbance at 450 nm for each well was measured using a microplate reader (Infinite M200).

### Clonogenic cell survival assay

For colony formation assay, 1000 viable cells were seeded onto 6-well plates. The cells were treated with 20 nM diABZI or DMSO for 2 h, and then exposed to 0, 2 and 4 Gy of  $\gamma$ -ray irradiation. The cells were incubated in complete medium for 14 days to form colonies. The colonies were fixed with methanol for 15 min and then stained with Giemsa stain solution (Solarbio) for 30 min. Images of the plates were taken to analyze the colony, and count the colony using Image J software.

### Apoptosis assay

Cell apoptosis was analyzed using Annexin V-FITC kit (BD) according to the manufacturer's protocol. In brief, cells ( $3 \times 10^5$  cells/well) were seeded onto 6-well plates overnight, and then treated with 20 nM diABZI or DMSO for 2 h, followed by exposure to 0, 2 and 4 Gy of  $\gamma$ -ray irradiation. After 48 h incubation, the cells were stained with Annexin V-FITC and PI, and then analyzed by flow cytometry (BD FACSCelesta, BD Biosciences).

### Quantitative real-time PCR (qRT-PCR)

Total RNA in cells was isolated using TRIzol reagent (Invitrogen), and reversely transcribed into cDNA using poly (A)-tailed total RNA and reverse transcription primer with ImPro-II Reverse Transcriptase (Promega) following the manufacturer's instructions. The qRT-PCR was carried out using Fast Start Universal SYBR Green Master (Rox) (Roche Diagnostics GmbH Mannheim)

according to the protocol. The primers used in this study were as follows:

cGAS forward, 5'-AAGGCCTGCGCATTCAAAAAC-3', and reverse, 5'-TGTGAGAGAAGGATAGCCGC-3';  
 STING forward, 5'-AGCATTACAACAACCTGCTACG-3', and reverse, 5'-GTTGGGGTCAGCCATACTCAG-3';  
 TBK1 forward, 5'-TGGGTGGAATGAATCATCTACGA-3', and reverse, 5'-GCTGCACCAAAATCTGTGAGT-3';  
 IRF3 forward, 5'-ACAGCAGGAGGATTTCCGG-3', and reverse, 5'-TATGTGGTTCGTGAGGGTC-3';  
 NF- $\kappa$ B p65 forward, 5'-CCCTGAGGCTATAACTCGC-3', and reverse, 5'-AGAAGTCCATGTCCGCAAT-3';  
 BCL2-Associated X (BAX) forward, 5'-CGAACTGGACAGTAACATGGAG-3', and reverse, 5'-CAGTTTGTGGCAAAGTAGAAA-3';  
 Caspase-3 forward, 5'-CCAAAGATCATACTGGAAGCG-3', and reverse, 5'-CTGAATGTTTCCCTGAGGTTTG-3';  
 GAPDH forward, 5'-GGAGCGAGATCCCTCCAAAAT-3', and reverse, 5'-GGCTGTTGTCATACTTCTCATGG-3'.

## Measurement of cGAMP

cGAMP in cell lysate was measured by human cGAMP ELISA Kit (Shanghai Tongwei) according to the protocol. The result was normalized by total protein concentration in cells.

## Immunofluorescence staining

H460 cells ( $1 \times 10^5$  cells/well) were seeded onto chambered slides and cultured overnight. After pretreatment with 10  $\mu$ M A12B4C3 (an inhibitor of polynucleotide kinase/phosphatase) for 2 h, the cells were exposed to 0, 2 and 4 Gy of  $\gamma$ -ray irradiation, and cultured for 6 h. Subsequently, the cells were sequentially fixed and penetrated with 4% paraformaldehyde for 30 min and 0.1% Triton X 100 for 30 min, respectively. The cells were then blocked with 20% normal

goat serum. After 1 h, the cells were incubated with antiphospho-histone H2AX (ser139) antibody (1:400 dilution, Cell Signaling Technology) and then with Alexa Fluor 555 anti-mouse IgG (1:600 dilution, Cell Signaling Technology). Finally, the cells were counterstained with DAPI (Solarbio), and images were collected with a Leica TCS SP8 confocal microscope.

## Statistical analysis

Each experiment was carried out independently at least three times, and the results expressed as the mean  $\pm$  standard deviation. A Student's *t*-test was used to analyze the statistical significance and  $p < 0.05$  was defined as statistically significant.

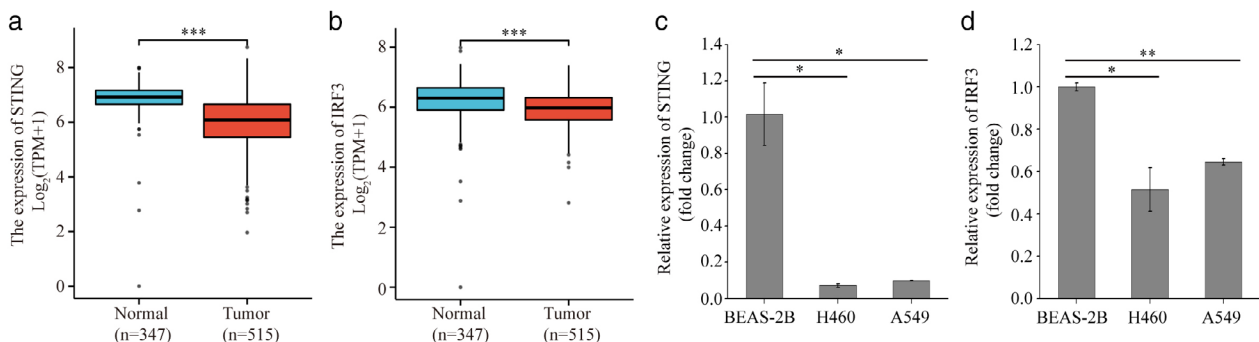
## RESULTS

### Low expression of cGAS-STING signaling pathway genes in NSCLC

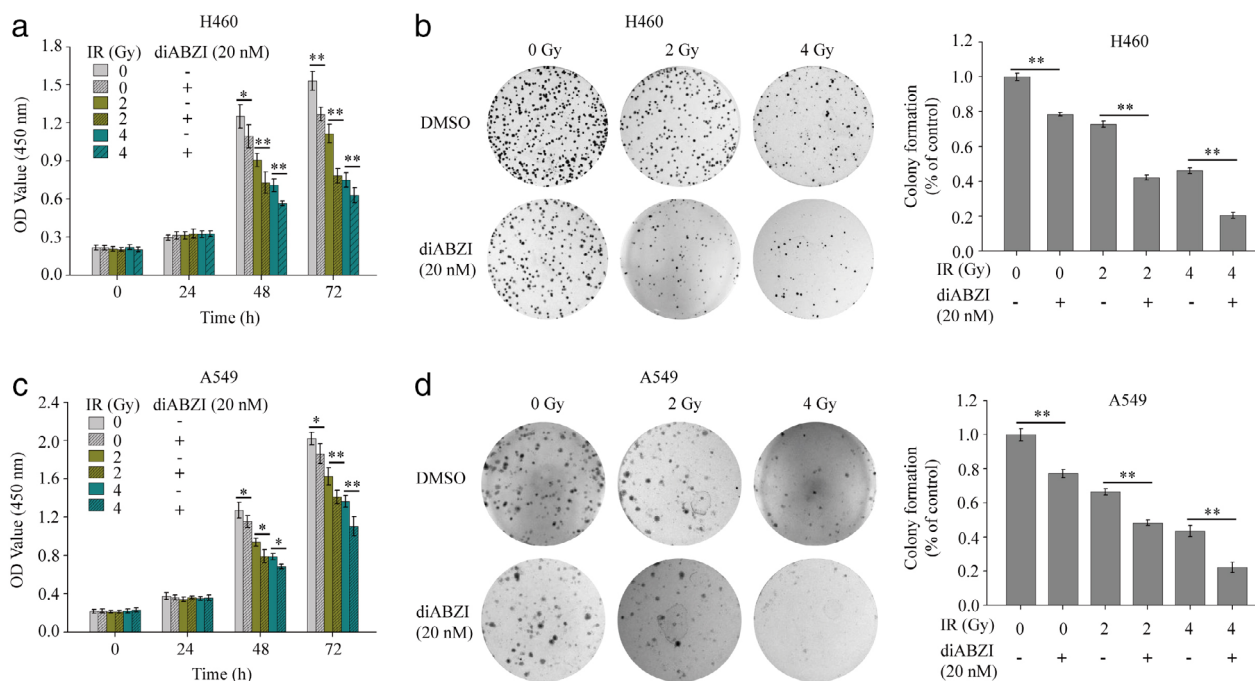
In NSCLC, the expression levels of STING and IRF3, signaling molecules in the cGAS-STING pathway, were lower in lung adenocarcinoma compared with normal lung tissue (Figure 1a and b). The lower levels of STING (Figure 1c) and IRF3 (Figure 1d) were also detected in NSCLC, H460 and A549 cells.

### cGAS-STING pathway activator enhances the inhibitory effect of irradiation on NSCLC cells

According to a previous study,<sup>8</sup> the small molecule diABZI was able to activate the cGAS-STING pathway. We thus used diABZI to reinforce the activation of cGAS-STING pathway and then investigate its inhibitory effect of irradiation on NSCLC cells. The proliferation of NSCLC cells was studied through CCK-8 and colony-formation assays, and



**FIGURE 1** Expression levels of cGAS-STING signaling pathway genes in NSCLC. The levels of (a) STING and (b) IRF3 in lung adenocarcinoma compared to normal lung tissue using public datasets (TCGA and GTEx datasets). The mRNA levels of (c) STING and (d) IRF3 in normal cells (BEAS-2B) and NSCLC cells (H460 and A549) analyzed by qRT-PCR. The data from three independent experiments are presented as mean  $\pm$  standard deviation. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$



**FIGURE 2** Effect of cGAS-STING pathway activator on the radiosensitivity of NSCLC cells. Cells pretreated with 20 nM diABZI or DMSO were treated with IR (0, 2 and 4 Gy), and cell proliferation of (a, b) H460 and (c, d) A549 cells were measured using CCK-8 and colony formation assays. (a, c) CCK-8, and (b, d) colony formation assays. In (b) and (d) the left panel is representative images of colonies and the right panel shows quantification of colonies. Each experiment was repeated at least three times. \* $p < 0.05$ ; \*\* $p < 0.01$

the results are shown in Figure 2. Compared to the control cells, the proliferation of both H460 (Figure 2a and b) and A549 cells (Figure 2c, d) was obviously inhibited by diABZI. It was also found that the proliferation of NSCLC cells treated with diABZI and IR (2 or 4 Gy) was further significantly inhibited compared with that of the cells only irradiated at 2 or 4 Gy. Moreover, the inhibitory effect of diABZI combined with a low dose of IR (2 Gy) on the proliferation of NSCLC cells was closer to that of a higher dose of IR (4 Gy). These results indicated that diABZI could effectively sensitize NSCLC cells to IR, namely the increased activation of cGAS-STING pathway could enhance the radiosensitivity of NSCLC cells.

Further analysis revealed that diABZI did not exhibit a significant inhibitory effect on the proliferation of the irradiated H460 (Figure 2a) and A549 cells (Figure 2c) until 48 h after IR (0, 2 or 4 Gy). Based on this result, the apoptosis of NSCLC cells was analyzed at 48 h after IR.

### Increasing activation factor of cGAS-STING pathway enhances the inhibitory effect of irradiation on NSCLC cells

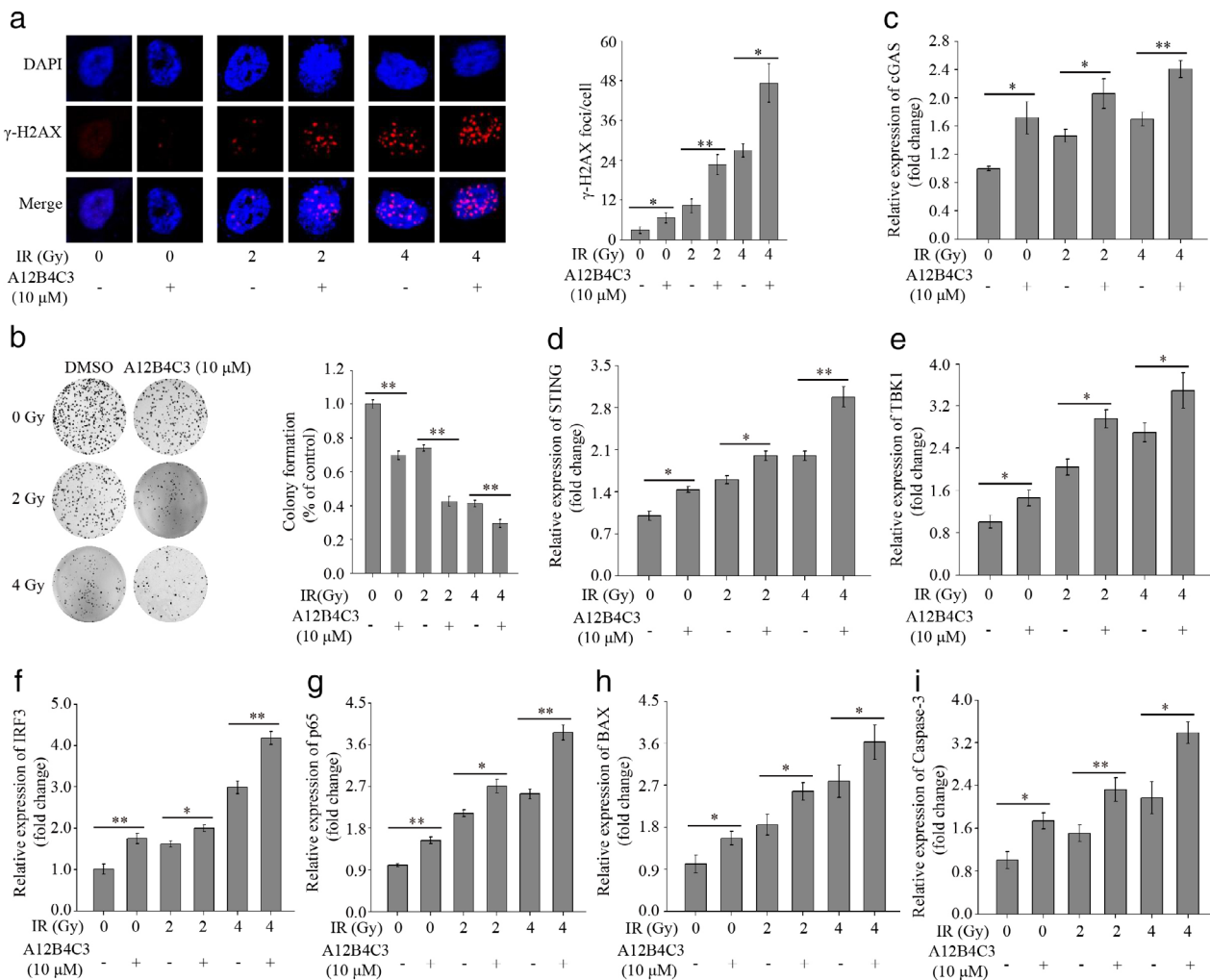
To further verify that the increased activation of cGAS-STING pathway could enhance radiosensitivity of NSCLC cells, we used an inhibitor of human polynucleotide kinase/phosphatase (hPNKP), A12B4C3, to inhibit DNA repair,<sup>18</sup> generating more DSBs (activation factor of cGAS-STING

pathway), and the results are shown in Figure 3a. It was found that A12B4C3 significantly increased DSBs in both the control and irradiated H460 cells, consistent with a previous study.<sup>18</sup> The increased DSBs led to the inhibition of cell proliferation, which could be deduced from the result that the colony formation rate of H460 cells treated with A12B4C3 and IR (0, 2 or 4 Gy) was significantly lower than that of the corresponding control H460 cells, as shown in Figure 3b. Furthermore, the increased DSBs reinforced the activation of cGAS-STING pathway, which was inferred from the increased levels of relevant signaling molecules of cGAS-STING pathway, cGAS (Figure 3c), STING (Figure 3d), TBK1 (Figure 3e), IRF3 (Figure 3f) and NF- $\kappa$ B p65 (p65) (Figure 3g), in H460 cells treated with A12B4C3 and IR (0, 2 or 4 Gy), compared with the corresponding control cells. In addition, the apoptosis markers, BAX (Figure 3h) and caspase-3 (Figure 3i), were also upregulated in H460 cells treated with A12B4C3 and IR. These results suggested that the increased DNA damage could trigger a higher activation of cGAS-STING pathway and cause a promotion of cell apoptosis, leading to enhanced radiosensitivity of NSCLC cells.

### Increased activation of cGAS-STING pathway promotes apoptosis of irradiated NSCLC cells

To reveal the underlying mechanism of the increased activation of cGAS-STING pathway enhancing the radiosensitivity



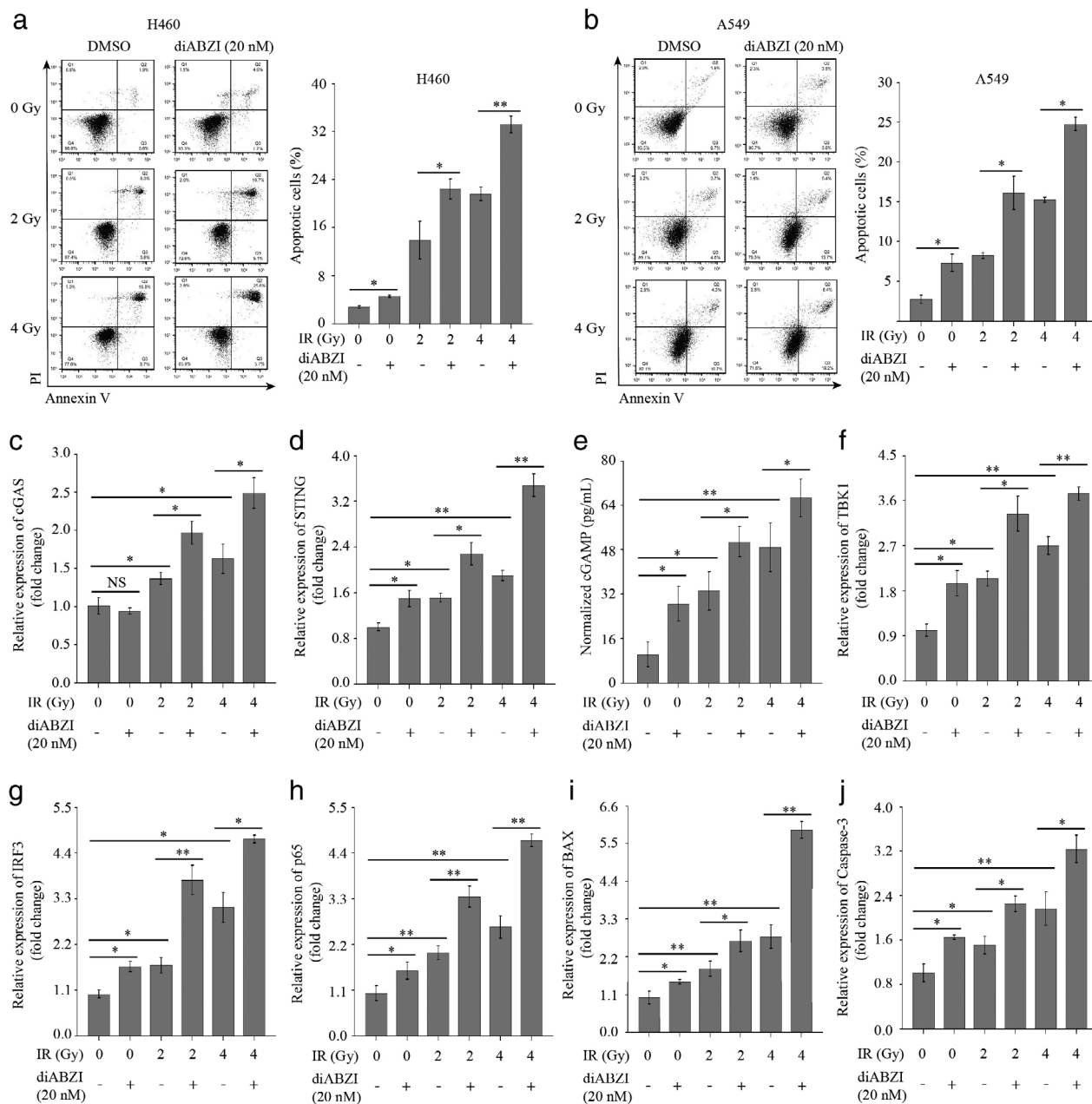


**FIGURE 3** Effect of increasing activation factor of cGAS-STING pathway on the radiosensitivity of NSCLC cells. (a) DSB induction in H460 cells by A12B4C3 and/or IR. H460 cells pretreated with A12B4C3 (10 μM) or DMSO were irradiated with  $\gamma$ -ray (0, 2 and 4 Gy). After incubation with antibodies against  $\gamma$ H2AX and Alexa Fluor 555 anti-mouse IgG, the cells were counterstained with DAPI. Representative images of  $\gamma$ H2AX foci staining (400 $\times$  magnification) (left panel) and quantification of  $\gamma$ H2AX foci (right panel) in H460 cells are shown. (b) H460 cells pretreated with 10 μM A12B4C3 or DMSO were treated with IR (0, 2, and 4 Gy) and analyzed using a colony-formation assay. Representative images (left panel) and quantification of colonies (right panel) are shown. (c–i) The mRNA levels of relevant signaling molecules in H460 cells. (c) cGAS, (d) STING, (e) TBK1, (f) IRF3, (g) p65, (h) BAX and (i) Caspase-3. The data from three independent experiments are presented as mean  $\pm$  standard deviation. \* $p < 0.05$ ; \*\* $p < 0.01$

of NSCLC cells, we measured the apoptosis in NSCLC cells. For this exploration, NSCLC cells (H460 and A549 cells) were irradiated with increasing doses after pretreatment with diABZI. Different cellular physiological states were assessed at 48 h after IR using Annexin V-FITC/PI double staining and flow cytometry, and the results are shown in Figure 4. Analysis of the apoptotic cells (early apoptotic cells plus late apoptotic cells) (Figure 4a, b) indicated that diABZI significantly increased the cell apoptosis rate in the irradiated H460 cells (2 Gy, 13.9%  $\pm$  3.2% vs. diABZI+2 Gy, 22.4%  $\pm$  1.7%, and 4 Gy, 21.6%  $\pm$  1.1% vs. diABZI+4 Gy, 33.2%  $\pm$  1.4%) and A549 cells (2 Gy, 8.2%  $\pm$  0.4% vs. diABZI+2 Gy, 16.1%  $\pm$  2.1%, and 4 Gy, 15.2%  $\pm$  0.3% vs. diABZI+4 Gy, 24.8%  $\pm$  0.9%). Moreover, the apoptosis rate of H460 cells treated with diABZI and a low dose of IR (2 Gy) was comparable to that of cells only receiving a

higher dose of IR (4 Gy), as shown in Figure 4a. A similar result was also found in the apoptosis rate of A549 cells (Figure 4b). These results manifested that the reinforced activation of cGAS-STING pathway enhanced the radiosensitivity of NSCLC cells by promoting apoptosis.

To prove that diABZI enhanced the radiosensitivity of NSCLC cells through reinforcing activation of cGAS-STING pathway, we measured the expression levels of relevant signaling molecules (Figure 4 and Figure S1). It was found that in comparison with the control cells, the expression levels of cGAS (Figure 4c), STING (Figure 4d), cGAMP (Figure 4e) and downstream molecules, TBK1 (Figure 4f), IRF3 (Figure 4g) and p65 (Figure 4h), significantly increased in H460 cells that only received IR, and increased with dose. A similar trend was also found in the expression levels of relevant signaling molecules in A549 cells (Figure S1). These



**FIGURE 4** Analysis of the mechanism of radiosensitization in NSCLC cells. (a, b) Apoptosis analysis of NSCLC cells using Annexin V-FITC/PI double staining and flow cytometry. (a) H460 and (b) A549 cells pretreated with 20 nM diABZI or DMSO were irradiated with  $\gamma$ -ray (0, 2, and 4 Gy) and the apoptosis was assessed at 48 h after IR. Representative images (left panel) and percentages of apoptotic cells (early apoptotic cells plus late apoptotic cells) (right panel) are shown. (c–j) Expression levels of relevant signaling molecules in H460 cells. The mRNA levels of (c) cGAS and (d) STING were analyzed by qRT-PCR. (e) cGAMP level in H460 cell lysate was measured by human cGAMP ELISA kit and normalized by total protein concentration. The mRNA levels of (f) TBK1, (g) IRF3, (h) p65, (i) BAX and (j) Caspase-3 in H460 cells were analyzed by qRT-PCR. All tests were repeated at least three times and the data are represented as mean  $\pm$  standard deviation. NS, not significant; \* $p < 0.05$ ; \*\* $p < 0.01$

implied that IR activated the cGAS-STING pathway in NSCLC cells, which has been also reported in other cancer cells.<sup>8,13</sup>

When treated with diABZI and IR (2 or 4 Gy), H460 cells expressed higher levels of signaling molecules, cGAS (Figure 4c), STING (Figure 4d), cGAMP (Figure 4e) TBK1 (Figure 4f), IRF3 (Figure 4g) and p65 (Figure 4h), compared with the irradiated cells. A similar trend was

also found in A549 cells (Figure S1). The results showed that diABZI could reinforce the activation of the cGAS-STING pathway in both irradiated H460 and A549 cells. Moreover, the increased expression of IRF3 and p65 in cells treated with diABZI and IR would directly induce the production of IFNs and other cytokines,<sup>19,20</sup> leading to further inhibition of cell proliferation, as shown in Figure 2. In addition, diABZI also upregulated protein

levels of phospho-IRF3 (pIRF3) and phospho-p65 (pp65) in the irradiated cells (Figure S2), further demonstrating reinforced downstream activation of the cGAS-STING pathway. Furthermore, the expression levels of the apoptosis markers, BAX (Figure 4i and Figure S1F) and Caspase-3 (Figure 4j and Figure S1G), also significantly increased in cells treated with diABZI and IR, implying a promotion of apoptosis, which was consistent with the results of cell apoptosis (Figure 4a, b). Together with the upregulated expression of BAX (Figure 3h) and caspase-3 (Figure 3i) in cells treated with A12B4C3 and IR, we thus concluded that the increased activation of cGAS-STING pathway could enhance the radiosensitivity of NSCLC cells by promoting cell apoptosis.

## DISCUSSION

Radiotherapy is one of the primary treatment options for NSCLC patients, and radioresistance is still a major challenge to improve the therapeutic outcomes.<sup>4,5</sup> A great deal of effort has been devoted to seeking effective radiosensitizing approaches for the treatment of NSCLC. Recently, activation of the cGAS-STING pathway has exhibited potential to improve the efficacy of immune checkpoint inhibitors and to stimulate anticancer immune response, becoming an attractive therapeutic target.<sup>8–10,21</sup> However, the relationship between activation of the cGAS-STING pathway and radiosensitization of NSCLC cells is still unclear.

In this study, we found that the expression levels of cGAS-STING pathway genes were relatively low, including STING, in NSCLC cells. Hence, we used an agonist of STING, diABZI, to increase the activation of cGAS-STING pathway to investigate whether reinforced activation of cGAS-STING pathway could enhance the radiosensitivity of NSCLC cells. In addition, we also used A12B4C3, a potent inhibitor of hPNKP, to inhibit DNA repair, generating increased DSBs (activation factor) and then to further prove the effect of the increased cGAS-STING pathway activation on the radiosensitization of NSCLC cells.

Our results showed that diABZI effectively sensitized NSCLC cells (H460 and A549 cells) to IR. Apoptosis analysis showed that diABZI could significantly promote apoptosis in irradiated NSCLC cells. Growing evidence has proved that based on the induced DSBs, IR activates the cGAS-STING pathway,<sup>19,22</sup> which was also verified through higher levels of the relevant signaling molecules in irradiated NSCLC cells in this study. Moreover, the increased DSBs induced by A12B4C3 could trigger a higher activation of the DNA-sensing cGAS-STING pathway, accompanied with higher radiosensitivity of NSCLC cells. In summary, the increased activation of cGAS-STING pathway could significantly inhibit cell proliferation through promoting apoptosis in NSCLC cells.

Our findings indicate that diABZI may be a promising radiosensitizer for NSCLC cells, and further studies should

be directed towards the influence of diABZI on the radiosensitivity of NSCLC cells in vivo to provide strong support for this conclusion. More importantly, our findings reveal that targeting the activation of cGAS-STING pathway (not limited to a single molecular target) offers a new option to enhance the efficacy of radiotherapy in the treatment of NSCLC.

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## CONFLICT OF INTEREST

No authors report any conflict of interest.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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