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G3BP1 Depletion Increases Radiosensitisation by Inducing Oxidative Stress in Response to DNA Damage

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Abstract. *Background: RAS GTPase-activating protein-binding protein (G3BP1) is an RNA-binding protein that is essential for assembling stress granules. Many functions related to the survival and progression of cancer have been reported. The current study aimed to investigate the role of G3BP1 in radio-sensitisation of cancer cells. Materials and Methods: Radiation sensitivity and chemosensitivity were analysed in A549 and H460 cells transfected with G3BP1 siRNAs, and N-acetyl-L-cysteine (NAC) was used to elucidate the involvement of reactive oxygen species (ROS). Results: G3BP1 depletion sensitised lung cancer cell lines to radiation, and the effect was related to ROS. G3BP1 depletion impaired the intracellular ROS scavenging system and NAC abolished the radiation-sensitive phenotypes caused by G3BP1 depletion. Conclusion: The study suggested G3BP1 as a promising target for radio- and chemosensitisation of lung cancer.*

Radiation therapy is a central treatment strategy for various types of cancer (1, 2). However, treatment efficiency may be compromised due to the radioresistance of cancer cells, and thus the response of patients to radiotherapy may vary considerably. Therefore, increasing sensitivity of cancer cells to radiation, with minimal effect on normal cells, has long been intended in radiotherapy (3-5).

Stress granules (SGs) are cytoplasmic mRNA complexes formed under various stress conditions such as oxidation (sodium arsenite), heat shock, hypoxia, and UV irradiation. They stall initiation of global translation for defence against these stress conditions, thereby inhibiting apoptosis (6-8). Several studies have suggested the targeting of SGs as a therapeutic approach in cancer (8-12). SGs have been widely found in cancer tissues, especially in an hypoxic microenvironment, which is the major cause of radioresistance (8-10). In the context of radiosensitisation, SGs might be a valuable target since hypoxia condition in activated SGs are closely related to that in radioresistance (10).

RAS GTPase-activating protein-binding protein (G3BP1) is an RNA-binding protein that is essential in SG formation as an SG-nucleating protein (13-15); it is overexpressed in various tumour tissues, and is related to tumour progression and metastasis (9, 15-18). As SGs may have functions related to radiosensitisation as mentioned above, we investigated whether G3BP1, which is a key factor in SG formation, is related to radiosensitisation.

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Materials and Methods

Cell lines. A549 and H460 non-small cell human lung cancer lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI medium (Welgene, Gyeongsan, Korea) supplemented with 10% foetal bovine serum (J R Scientific, Woodland, CA, USA) and 1 % (w/v) antibiotics (Welgene) at 37˚C in a humidified atmosphere containing 5% CO_2.

Small interfering RNAs (siRNAs), plasmids, and transfection. The siRNAs were synthesised, except for the control siRNA that was purchased from Bioneer (Daejeon, Republic of Korea). The sequences used were as follows: *G3BP1*, #1: 5'-CCAAGAUGAGGUCUUU GGUGGGUUUUU-3'; #2: 5'-GCGCAUUAACAGUGGUGGGAAA UUAUU-3'. Cells were transfected with siRNA at a final concentration of 20 nM, using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) in Opti-MEM (Invitrogen). The knockdown of mRNA by siRNA was confirmed by reverse transcription PCR. cDNA was synthesised using iScript cDNA synthesis kit (Bio-Rad, Carlsbad, CA, USA) and reverse transcription-polymerase chain reaction (RT-PCR) was performed using Maxime RT PreMix (Oligo dT) (iNtRON, Seongnam, Republic of Korea). The following primers were used:

G3BP1: 5'-CCA TTT GAA TCC AAT CCC CCA-3' and 5'-GAG AAG CCT AGT CCC CTG CT-3'; β-actin: 5'-GGA CTT CGA GCA AGA GAT GG-3' and 5'-AGC ACT GTG TTG GCG TAC AG-3'.

Clonogenic assay. Cells were transfected with siRNA, incubated for 24 h, thereafter seeded at a density of 2×10^3 in 60-mm dishes, incubated again for 24 h, and subsequently irradiated with 2 or 4 Gy using a Gammacell 3000 Elan irradiator (^{137}Cs γ-ray source; MD S Nordin, ON, Canada). Incubation of cells continued until visible colonies were formed. The colonies were then fixed with 10% formaldehyde and stained with crystal violet. Survival rate was calculated as: number of colonies under irradiated condition/number of colonies under non-irradiated condition. To evaluate chemosensitisation by G3BP1 depletion, clonogenic assay was performed with the same method measuring radiosensitivity. Cells were treated with 40 nM of doxorubicin after low-density seeding for 6 h, then after replacement of the medium, cells were incubated until visible colonies were formed.

Fluorescence microscopy. Cells grown on glass coverslips placed in a 6 well plate were transfected with siRNA, incubated for 24 h, then irradiated with 4 Gy and further incubated for 6 h. For immunostaining, cells were fixed with 10% formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature, washed with 0.1% Triton X-100 in PBS (PBS-T), incubated in PBS-T for 10 min, and incubated with blocking solution (3% skim milk in PBS). The slides were then incubated with primary antibodies against γ-H2A histone family member X (H2AX) for 2 h (Abcam, Cambridge, UK). Subsequently, the slides were washed with PBS, incubated with Alexa 488- or 555-conjugated secondary antibodies for 1 h (Invitrogen), washed again, and mounted using VECTASHIELD Mounting Medium with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). The stained cells were visualised under a fluorescence microscope (Axio Imager M2; Carl Zeiss AB, Stockholm, Sweden).

Immunoblotting. Cells were transfected with siRNA, irradiated after 24 h, further incubated for 24 h, then harvested and lysed with TNN buffer (120 mM NaCl, 40 mM Tris-HCl, pH 8.0, 0.5% NP-40) supplemented with a protease inhibitor cocktail (GenDEPOT, Barker, TX, USA). The purified proteins were subjected to polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and analysed using primary antibodies against G3BP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), poly (ADP-ribose) polymerase (PARP; Cell Signaling Technology, Stockholm, Sweden), β-actin (Santa Cruz Biotechnology), caspase-3 (Cell Signaling Technology), γ-H2AX (phospho Ser139; Millipore, Bedford, MA, USA), sulfiredoxin 1 (SRX1; Santa Cruz Biotechnology), glutathione peroxidase 1 (GPX1; Biodesign, Kennebunk, ME, USA), superoxide dismutase 2 (MnSOD, Biodesign), peroxiredoxin 1 and 2 (PRX1, PRX2; Younginfrontier, Seoul, Republic of Korea). After incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Bethyl Laboratories, Montgomery, TX, USA), the proteins were detected by a chemiluminescence detection system (Santa Cruz Biotechnology).

Flow cytometry. Apoptosis, cell cycle distribution, and intracellular reactive oxygen species (ROS) levels were analysed using flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA). Cells were transfected with siRNA, irradiated with 4 Gy after 24 h, further incubated for 24 h before eventually prepared for flow cytometric analysis (19). For experiments including ROS scavenger treatment, cells were treated with 5 mM *N*-acetyl-L-cysteine (NAC; Sigma-Aldrich, St. Louis, MO, USA) for 2 h before radiation exposure. For cell-cycle analysis, ethanol-fixed cells were incubated with RNase and subsequently stained with 50 ng/ml propidium iodide (Sigma-Aldrich). Cell-cycle profile was analysed using the FL-2 channel. For apoptosis assays, harvested cells were stained with fluorescein-5-isothiocyanate (FITC)-conjugated annexin V antibody, as per the manufacturer's protocol (BD Pharmingen, San Diego, CA, USA), and then analysed by flow cytometry using FL-1 channel. ROS levels were measured by incubating the cells with 2',7'-dichlorofluorescein diacetate (DCF-DA; 10 μM; Sigma-Aldrich) for 30 min. Cells were harvested with trypsin, and analysed in the FL-1 channel using FACSCalibur. For statistical analyses, each experiment was individually performed four times.

Comet assay. Alkaline comet assay was performed as per the manufacturer's protocol (Trevigen, MD, USA). Cells were transfected with siRNA for 24 h, exposed to radiation with 4 Gy and harvested with trypsin immediately or after 1, 3, and 6 h, then embedded (5×10^3 cells) in low-melting agarose and subsequently dropped onto slides. Samples were lysed, rinsed, and treated with alkali to unwind DNA, followed by electrophoresis with the electrophoresis starter kit (Trevigen). SYBR green-stained samples were analysed with a fluorescence microscope (Axio imager M2; Carl Zeiss AB). The tail moment was analysed with comet analysis software (Trevigen) from over 200 cells in each sample. Abnormal tail moments, with upper and lower 10% in each sample, were eliminated and the mean calculated. For statistical analyses, each experiment was individually performed thrice.

Statistical analyses. All data were analysed as the mean±SD. Statistical differences between two means were assessed using Student's *t*-test (unpaired, two-tailed). A value of $p\leq0.05$ was considered to be statistically significant.

Results

Silencing of G3BP1 reduced clonogenic survival in response to radiation and doxorubicin. To examine the role of G3BP1 in radio-sensitisation, we performed clonogenic survival assay with A549 and H460 cell lines in which G3BP1 was depleted before cell irradiation. As shown in Figure 1A, each siRNA (#1, #2) targeted G3BP1, and irradiation synergistically reduced clonal cell survival. Depletion of G3BP1 by siRNAs was confirmed by RT-PCR (Figure 1B). G3BP1 depletion with siRNA #1 and #2 reduced the survival rate after irradiation (4 Gy) from 60% to 10% in A549 cells and from 25% to 8% (siRNA#1) and 6% (siRNA#2) in H460 cells.

Next, we investigated the function of G3BP1 in chemosensitisation. G3BP1 depletion sensitised A549 and H460 cells to doxorubicin (Figure 1C). The survival rate after doxorubicin treatment was reduced by G3BP1 depletion from 70% to 10% (siRNA#1) and 15% (siRNA#2) in A549 cells and from 80% to 30% (siRNA#1) and 20% (siRNA#2) in H460 cells.

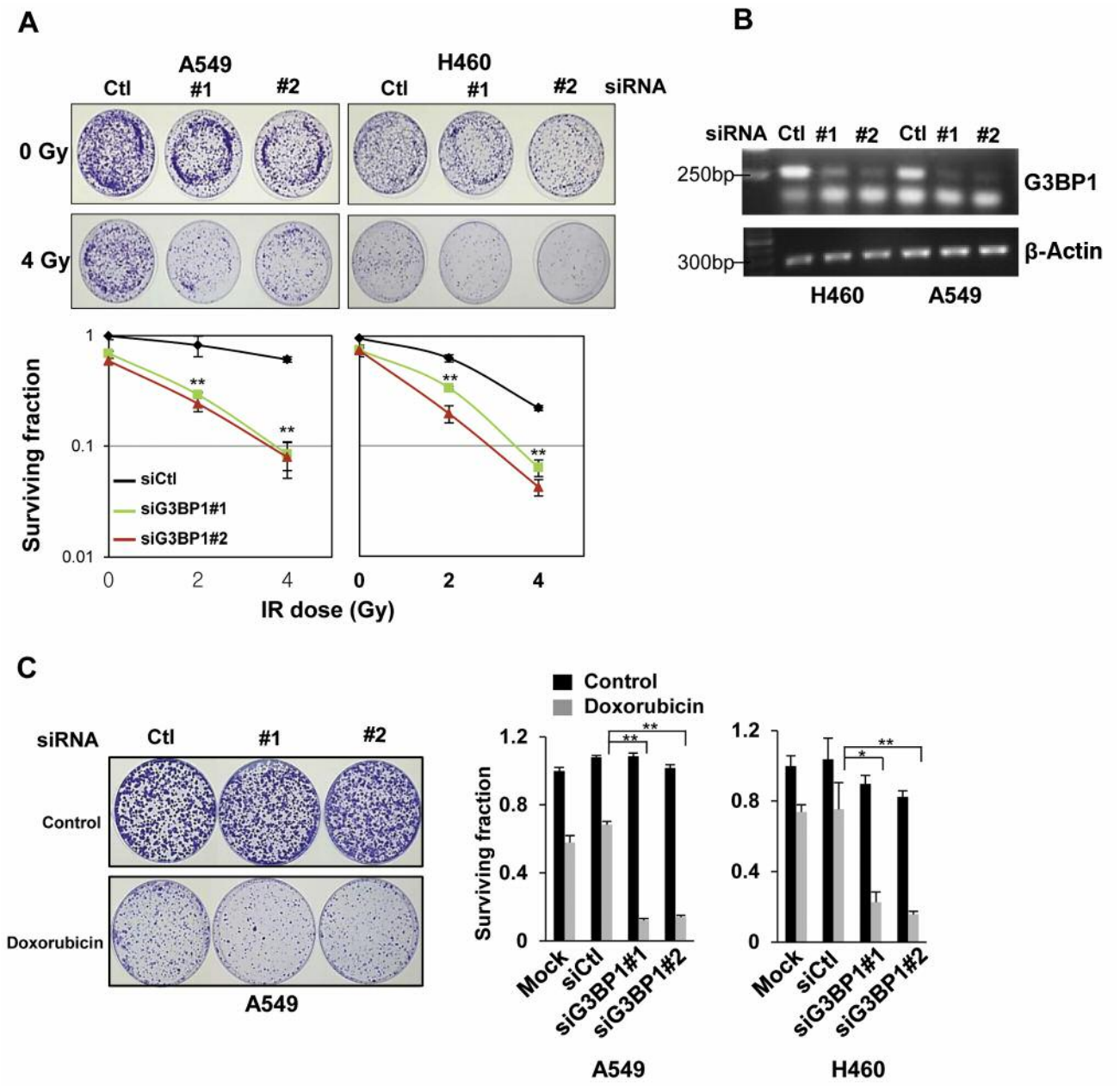

Figure 1. *RAS GTPase-activating protein-binding protein (G3BP1) depletion reduces the survival of non-small cell lung cancer cells under irradiation, and doxorubicin treatment. A549 and H460 cell lines were transfected with G3BP1 (#1 and #2) or control-siRNA, and then subjected to 4-Gy irradiation or doxorubicin treatment. The surviving fraction was determined by clonogenic assay. Colonies were stained with crystal violet and the survival fraction of each sample was calculated as the ratio of surviving colonies under each condition relative to that under the non-treated condition (A and C). Data are presented as means±SE from triplicates. Significantly different at *p<0.05 and **p<0.01. The assay was confirmed by three independent experiments having similar results. Depletion of G3BP1 by siRNA was confirmed by reverse transcription-polymerase chain reaction (B). siRNA, small interfering RNA; ctl, control, IR, irradiation.*

Silencing of G3BP1 increased radiation-induced DNA damage and apoptosis. To analyse the cellular phenotypes in radiosensitisation by G3BP1 depletion, we investigated DNA damage and apoptosis in A549 and H460 cells. Cells were irradiated after depletion by siRNAs for 24 h and all experiments were assayed at 12 h after irradiation. As shown in Figure 2A, PARP cleavage, γ-H2AX phosphorylation, and caspase-3 cleavage were synergistically increased due to the combination of G3BP1 depletion and irradiation in A549 cell line, thereby indicating the DNA damage and apoptosis

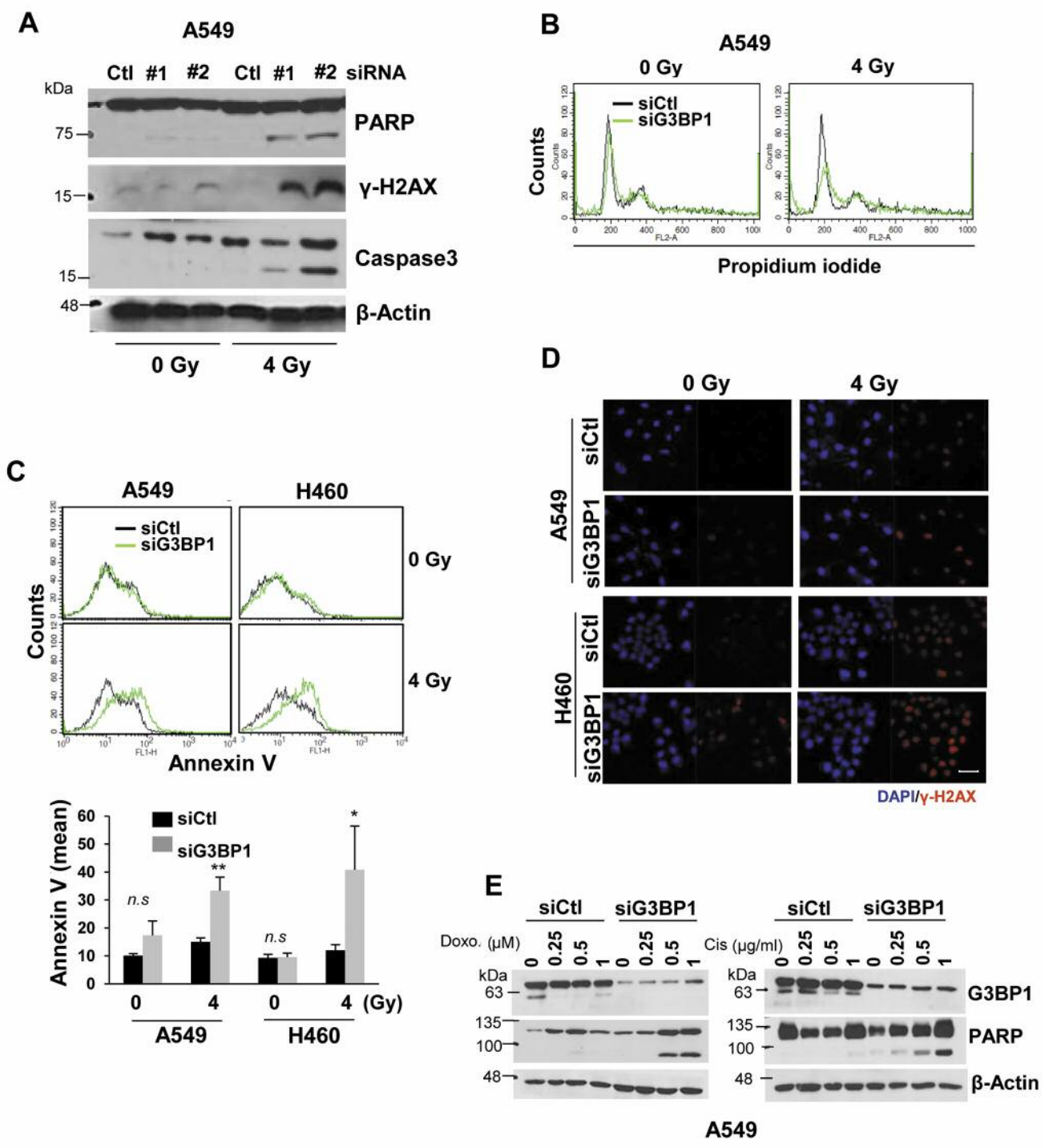

Figure 2. *RAS GTPase-activating protein-binding protein (G3BP1) depletion increases apoptosis of non-small cell lung cancer cells after irradiation or anticancer drug treatment. G3BP1- or control siRNA-transfected A549 and H460 cells were irradiated with 4 Gy, incubated for 24 h (A-C, E) or 6 h (D), and prepared for analysis. (A) A549 cell lysates were subjected to western blotting with antibodies against poly (ADP-ribose) polymerase (PARP), γ-H2A histone family member X (H2AX), caspase 3, and β-actin. (B) Cell-cycle profile of cells was analysed with flow cytometry. (C) Apoptotic (annexin-V-positive) cell profiles were analysed with flow cytometry (upper) and the percentages of annexin-V-positive apoptotic cells were determined by analysis of the histograms (lower). Data represent the mean±SEM of three independent experiments. Significantly different at *$p<0.05$ and **$p<0.01$; n.s.: not significantly different. (D) Representative photographs obtained by confocal microscopy at a magnification of 400×, showing the formation of γ-H2AX foci. Scale bar: 50 μm. (E) siRNA-transfected A549 cells were treated with anticancer drug for 24 h and western blotting was performed to analyse apoptosis through cleavage of PARP. siRNA, small interfering RNA; ctl, control.*

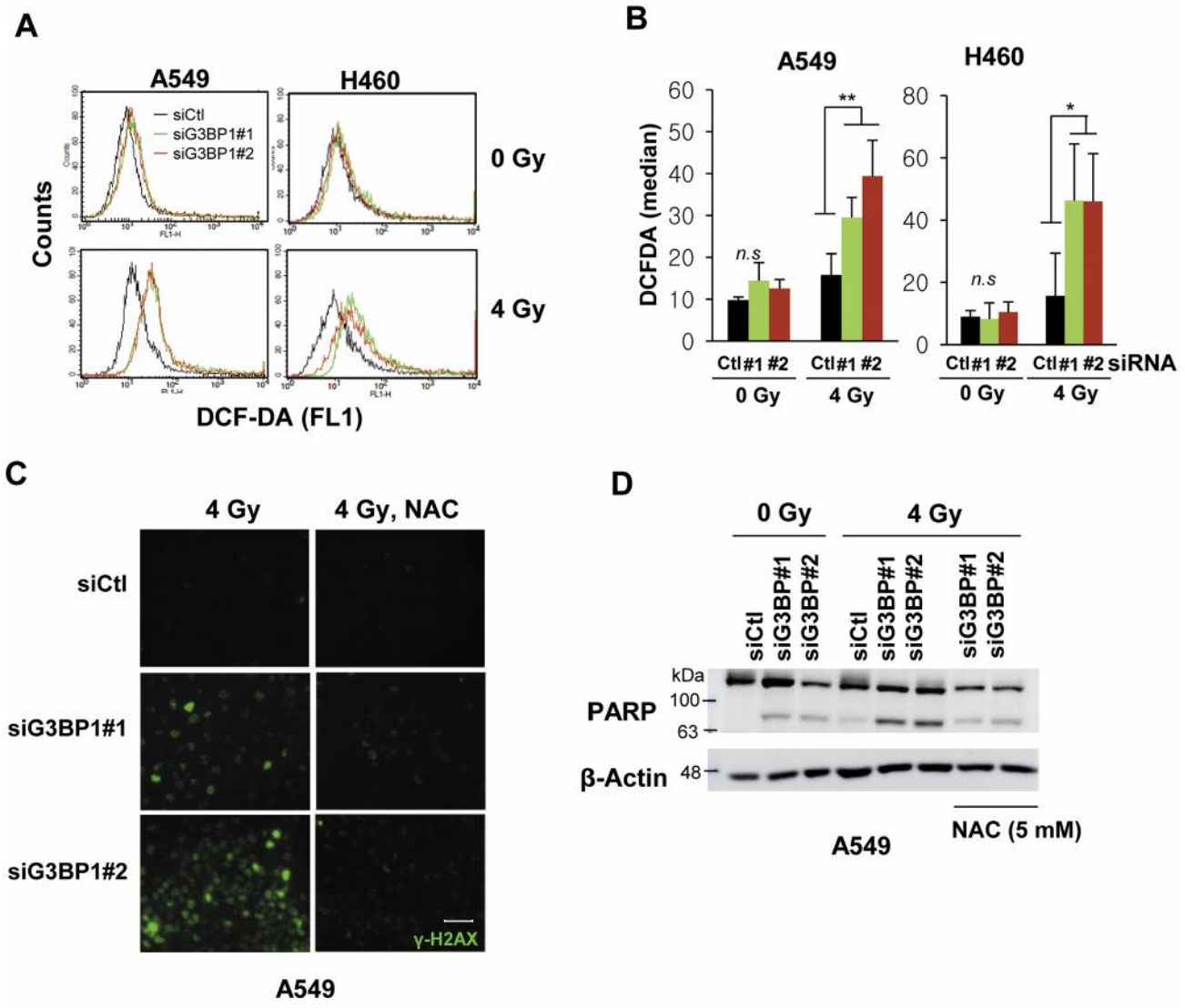

Figure 3. *RAS GTPase-activating protein-binding protein (G3BP1) depletion-mediated radiosensitisation depends on oxidative stress. G3BP1- and control siRNA-transfected A549 (A-D) and H460 (A, B) cells were irradiated with 4 Gy, incubated for 6 h (C), or 24 h (A, B, D), and eventually prepared for analysis. NAC was treated 2 h before irradiation. A: Levels of intracellular reactive oxygen species (ROS) were determined by measuring fluorescence of 2',7'-dichlorofluorescein diacetate (DCF-DA). The histogram of DCF-DA intensity is a representative of four independent experiments. B: ROS level in each experiment was quantified by median fluorescence. Data represent the mean±SEM of four independent experiments. Significantly different at *p<0.05 and **p<0.01; n.s.: not significantly different. C: Representative photographs, obtained by fluorescence microscopy at a magnification of 400×, showing the formation of γ-H2A histone family member X (H2AX) foci in A549 cells. Scale bar: 50 μm. D: siRNA-transfected A549 cells with/without irradiation under NAC-treated condition were analysed by western blotting for apoptosis. Ctl, Control; siRNA, small interfering RNA.*

mediated by radiation to be increased by G3BP1 depletion. Next, we analysed the cell-cycle distribution of A549 cell line under the same condition. The cell cycle pattern was changed, with increased sub G1 population in G3BP1-depleted and irradiated cells (Figure 2B). Apoptosis was quantitatively assessed by measuring annexin V with flow cytometry (Figure 2C). While the increase of apoptosis by G3BP1 depletion was not significantly enhanced in H460 cells and only slightly enhanced in A549 cells, apoptosis due to irradiation was synergistically increased by G3BP1 depletion in both A549 and H460 cells. DNA damage in response to irradiation was also increased by G3BP1 depletion, as shown in γ-H2AX signal in A549 and H460 cells (Figure 2D). We further analysed the effect of G3BP1 on chemosensitisation (using doxorubicin and cisplatin) by western blotting. G3BP1 depletion increased apoptosis after the treatment of A549 cell line with cisplatin and doxorubicin, as shown by the elevated levels of PARP cleavage in Figure 2E.

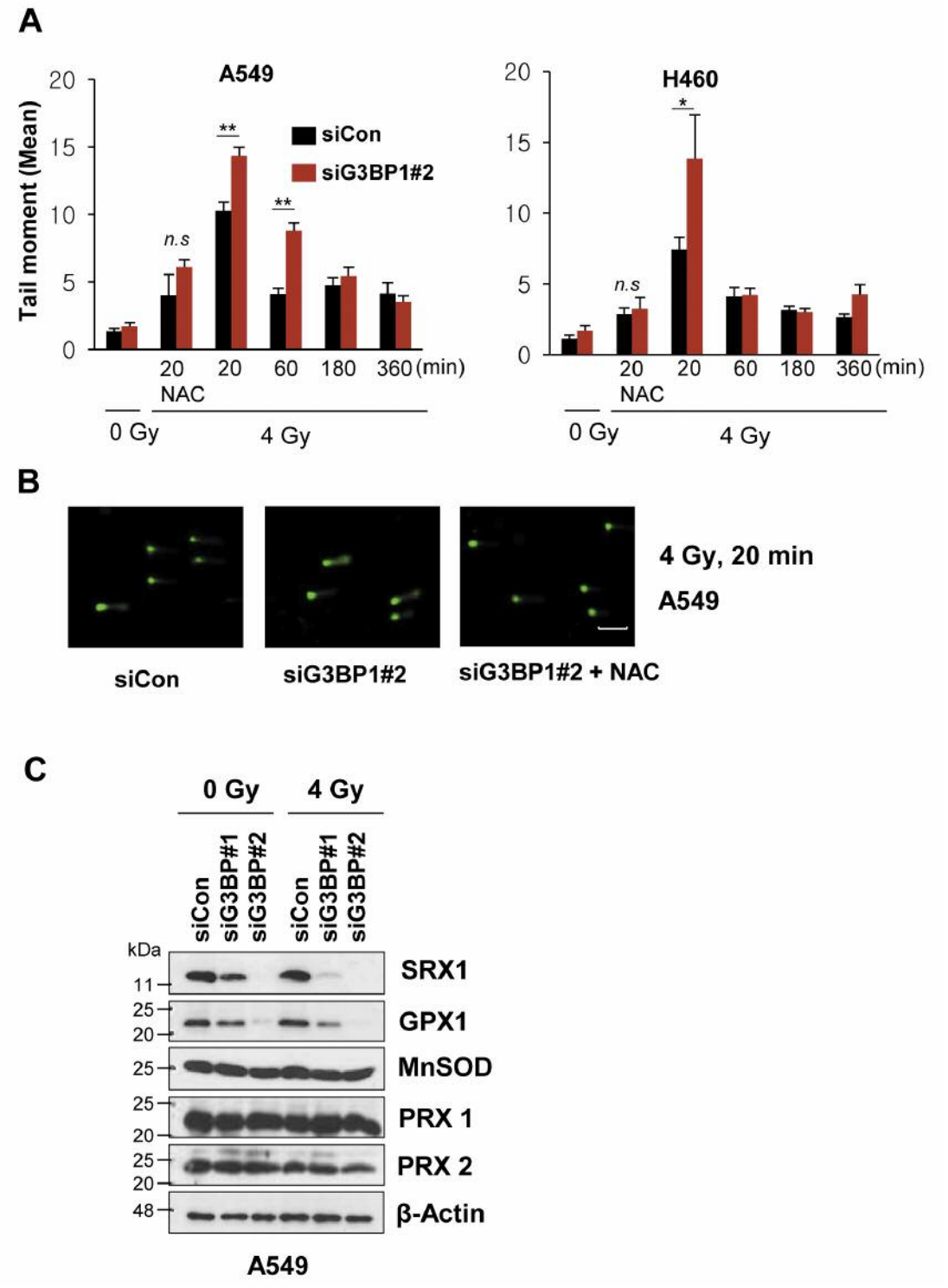

Figure 4. *RAS GTPase-activating protein-binding protein (G3BP1) depletion impairs DNA repair in a reactive oxygen species-dependent manner in response to radiation. (A) Comet assay was performed to measure unrepaired DNA damage caused by irradiation. G3BP1 siRNA-transfected cell lines were irradiated for the indicated times. Cells were also treated with N-acetyl-L-cysteine (NAC) before irradiation for 2 h. Data represent the mean±SEM of the tail moment from three independent experiments. Significantly different at *p<0.05 and **p<0.01. (B) Representative photographs of A549 cells were obtained by fluorescence microscopy at a magnification of 400×; scale bar: 50 μm. (C) The expression of main antioxidant enzymes, sulfiredoxin (SRX1), glutathione peroxidase 1 (GPX1), superoxide dismutase 2 (MnSOD), peroxiredoxin 1 and 2 (PRX1, PRX2) was analysed by western blotting in siRNA-transfected and irradiated A549 cells. siRNA, small interfering RNA.*

G3BP1 depletion increased radiosensitivity by regulating ROS production. Since SGs have been reported to play a role in the inhibition of apoptosis by reducing ROS (20), we analysed whether radiosensitisation by G3BP1 depletion might be related to ROS. siRNA-transfected A549 and H460 cells were irradiated and ROS was measured by detecting DCF-DA using flow cytometry (Figure 3A and B). Under non-irradiated condition, depletion of G3BP1 did not significantly affect intracellular ROS levels in either H460 or A549 cells. After irradiation for 3 h, ROS levels increased and became higher than that in G3BP1-depleted cells.

To analyse whether the defect of ROS scavenging upon G3BP1 depletion, under irradiated condition, directly affected cell radiosensitivity, we tested whether pre-treatment with NAC, a ROS scavenging drug, before irradiation would abrogate radiosensitivity induced by G3BP1 depletion. Firstly, DNA damage was analysed by staining siRNA-transfected cells with γ-H2AX in combination with irradiation and NAC treatment. As shown in Figure 3C, under irradiation, G3BP1-depletion induced γ-H2AX signal, representing DNA damage, which was significantly reduced by NAC treatment in A549 cells. We tested whether NAC affects G3BP1 depletion-mediated radiosensitisation by detecting PARP cleavage with western blotting. G3BP1-depletion-induced PARP cleavage in irradiated A549 cell line was reduced by NAC treatment, as shown in Figure 3D.

G3BP1 depletion impaired DNA repair and cellular ROS scavenging under irradiated conditions. To verify whether the function of G3BP1, related to the regulation of ROS and radiosensitisation, affected DNA strand break and repair after radiation damage, we performed a comet assay under the same conditions (Figure 4A and B). Under non-irradiated conditions, the tail moment in control siRNA-transfected cells and G3BP1 siRNA-transfected cells was similar to the basal level. After irradiation, DNA damage was induced promptly and repaired within 1 h. DNA breaks induced by radiation were increased further by G3BP1 depletion. We found the repair process of A549 cells to be delayed, after 1 h of irradiation. The repair activity after G3BP1 depletion in both cell lines recovered to the same levels as in control cells after 3 h. To determine whether ROS increase due to radiation was related to G3BP1 depletion-mediated increase of tail moment, NAC was applied before irradiation. ROS scavenging upon NAC treatment reduced DNA breaks due to radiation and DNA breaks due to G3BP1 depletion under irradiation were completely blocked by NAC treatment to a level similar to that of control cells.

Next, we tested the expression of representative antioxidant proteins in G3BP1-depleted A549 cells by western blotting (Figure 4C). We found GPX1 and SRX1 proteins to be reduced by G3BP1 depletion in both non-irradiated and irradiated A549 cells, while MnSOD and PRX proteins remained unchanged.

Discussion

Many types of cancer cells have increased levels of ROS, due to metabolic alteration, mitochondrial defect, or the tumour microenvironment (21), which causes them to be more vulnerable to oxidative insults and more dependent on antioxidant systems compared to normal cells (22). ROS also act as an important factor in radiotherapy, indirectly causing DNA damage (1, 2); some cancer types, especially cancer stem cells, have an increased antioxidant mechanism against oxidative stress (23). Therefore, targeting antioxidant mechanisms is a promising cancer-treatment strategy in radiotherapy (23-25). Lung cancer is the most prevalent cause of cancer-related deaths, and non-small cell lung cancer (NSCLC) is the most common type of lung cancer (26). Acquired resistance to radiotherapy or chemotherapy is the most common factor leading to treatment failure in patients with NSCLC. Therefore, studying the sensitisation of targets for radio- and chemotherapy is crucial for reducing their side-effects, and improving clinical outcomes of both in patients with NSCLC.

In the current study, we found that targeting G3BP1 caused radiosensitisation of A549 and H460 NSCLC cells in a ROS-dependent manner. Interestingly, while depletion of G3BP1 had only little cytotoxicity under non-irradiated conditions, it synergistically increased cell death under irradiation in a ROS-dependent manner. We also showed the cytotoxicity of anticancer drugs, namely doxorubicin and cisplatin, to be increased after G3BP1 depletion. These drugs induce ROS which also increases cytotoxicity in cancer cells (27-29); therefore, they may be expected to have a drug combination effect with G3BP1 inhibition.

We planned this study focusing on SGs that function to control free radicals; however, unlike under UV exposure (30), the SG were not assembled under ionising γ-radiation stress. This implied that G3BP1-depletion-related radiosensitisation may be independent of the function of SGs and some other cellular function may be associated with sensitisation to radiation. G3BP1 is known to play an important part in the regulation of SG dynamics as an RNA-binding protein. However, G3BP1 has also been implicated in cancer progression, invasion, and metastasis, either separately or in relation to SG formation. It contributes to cancer-related phenotypes by interacting with cancer-related proteins, RNA, or modulating SGs (15). Recently, it was shown to play a role in STING pathway via regulation of cGAS by priming DNA binding and activation (31). This study would also support the benefit of targeting G3BP1 in radiation therapy. Moreover, in cancer tissues, SGs are created due to hypoxic conditions and G3BP1 plays an important role in that; therefore, regulation of radiation sensitisation is expected to be more critical under *in vivo* conditions than *in vitro* ones.

In conclusion, the study has shown G3BP1 to be a candidate target of radio- and chemosensitisation. We found this sensitisation to depend on ROS production by radiation and reduction of antioxidant systems by G3BP1 depletion. Therefore, the deficiency of ROS-scavenging ability induced by G3BP1 depletion does not affect the cell in the normal state, although it increases cytotoxicity by increasing the production of free radicals upon irradiation. Further studies would be required to elucidate the molecular mechanisms regulating the antioxidant system in cancer cells.

Conflicts of Interest

The Authors declare no competing interest in regard to this study.

Authors' Contributions

H.J. Shin contributed to the experimental design, performed the experiments, analysed the data and wrote the article. E. Cho. and S. H. Kim performed the experiments and analysed the data. T. T. Than wrote the article and provided resources. E.R. Park, M.Y.Kim, and K.H.Lee. provided resources and analysed the data.

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