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# Silencing of poly(ADP-ribose) glycohydrolase sensitizes lung cancer cells to radiation through the abrogation of DNA damage checkpoint

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ABSTRACT

Poly(ADP-ribose) glycohydrolase (PARG) is a major enzyme that plays a role in the degradation of poly(-ADP-ribose) (PAR). PARG deficiency reportedly sensitizes cells to the effects of radiation. In lung cancer, however, it has not been fully elucidated. Here, we investigated whether PARG siRNA contributes to an increased radiosensitivity using 8 lung cancer cell lines. Among them, the silencing of PARG induced a radiosensitizing effect in 5 cell lines. Radiation-induced G2/M arrest was largely suppressed by PARG siRNA in PC-14 and A427 cells, which exhibited significantly enhanced radiosensitivity in response to PARG knockdown. On the other hand, a similar effect was not observed in H520 cells, which did not exhibit a radiosensitizing effect. Consistent with a cell cycle analysis, radiation-induced checkpoint signals were not well activated in the PC-14 and A427 cells when treated with PARG siRNA. These results suggest that the increased sensitivity to radiation induced by PARG knockdown occurs through the abrogation of radiation-induced G2/M arrest and checkpoint activation in lung cancer cells. Our findings indicate that PARG could be a potential target for lung cancer treatments when used in combination with radiotherapy.



## 1. Introduction

The formation of poly(ADP-ribose) (PAR) is rapidly induced by DNA damage. Once DNA damage, such as single-strand breaks (SSB) or double-strand breaks (DSB), has occurred, poly(ADP-ribose) polymerase1 (PARP1) is recruited to the sites of DNA damage and is catalytically activated to synthesize PAR from nicotine adenine dinucleotide (NAD+) on itself and on other repair and chromatin-remodeling factors [1]. In the case of SSB, a base excision repair (BER) protein, X-ray repair cross-complementing protein 1 (XRCC1), is recruited to the sites of DNA damage through a strong interaction with the PAR chain [2]. PAR formation caused by PARP1 activation is considered to be a key event in DNA repair, and thus several PARP1 inhibitors are now under development for cancer treatment in combination with DNA damaging agents or radiotherapy [1].

Recently, the importance of PAR degradation in DNA repair has also been considered. Poly(ADP-ribose) glycohydrolase (PARG) is the enzyme that is responsible for the degradation of PAR to free ADP-ribose [3,4]. While PARPs constitute a large family of 17 proteins encoded by different genes, PARG is encoded by a single gene but has multiple splicing variant types that are localized to distinct cellular components: full-length PARG111 is nuclear, PARG102 and PARG99 are cytoplasmic, and PARG65 is mitochondrial [5]. Nuclear and cytoplasmic isoforms of PARG have been shown to be recruited to sites of DNA damage and to be involved in the degradation of PAR under circumstances where DNA damage has been induced [6]. PARG-deficient cells showed a delay in PAR degradation and in SSB and DSB repair [7]. Moreover, radiosensitivity was significantly enhanced in PARG-deficient cells, compared with wild-type cells and PARP1-deficient cells [7,8]. Although these reports were based on studies in a limited number of cell types, PARG could be a promising target for cancer treatment in combination with radiotherapy.

Radiation therapy is a widely used for the treatment of both non-small cell lung cancer (NSCLC) and small cell lung cancer

*Abbreviations:* PARG, poly(ADP-ribose) glycohydrolase; PAR, poly(ADP-ribose); PARP, poly(ADP-ribose) polymerase; SSB, single-strand break; DSB, double-strand break; NAD, nicotine adenine dinucleotide; BER, base excision repair; XRCC1, X-ray repair cross-complementing protein 1; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer.

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(SCLC) [9,10]. However, intrinsic or acquired resistance is considered to be a main limitation of the efficacy of radiation in the treatment of lung cancer. Therefore, radiotherapy for patients with lung cancer is expected to benefit from combination with chemotherapy or the administration of a radiosensitizer. As described above, PARG seems to be a potential target for radiotherapy in the treatment of cancer. However, whether PARG inhibition is capable of enhancing the effect of radiotherapy in the treatment of lung cancer is not fully understood. To gain insight regarding this point, the present study investigated the radiosensitizing effects induced by PARG silencing via siRNA in 8 different cell lines. Furthermore, we also examined the mechanism underlying the sensitization to radiotherapy in lung cancer cells.

## 2. Material and methods

### 2.1. Cell lines

The human lung cancer cell lines PC-14, A427, NCI-H23 (H23), EBC1, NCI-H520 (H520), NCI-H2170 (H2170), SBC-3 and SBC-5, were used in this study. All the cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (GIBCO) and 1% antibiotic–antimycotic solution (Sigma–Aldrich) and were maintained at 37 °C in a humidified atmosphere containing 5% $CO_2$.

### 2.2. siRNA

For the siRNA experiments, the cells were transfected with 5 nM of siRNA specific for PARG (s16158, s16159 and s57868, Ambion) or a negative control (NC) using Lipofectamine RNAiMAX (Invitrogen). PARG siRNAs targeting exons 14, 18, and 9 were called PARG siRNA 1, 2 and 3, respectively. The cells were used at 48 h after transfection for each experiment.

### 2.3. Antibodies

For Western blotting, the following rabbit monoclonal or polyclonal antibodies were obtained from Cell Signaling Technology: antibodies to phospho-ATM, phospho-Chk1, phospho-Chk2 and PARP-1. Rabbit polyclonal anti-PAR (TREVIGEN), mouse monoclonal anti-PARG (Millipore), and mouse monoclonal β-actin (Sigma–Aldrich) were also used for Western blotting.

### 2.4. Western blotting

The cells were washed with ice-cold PBS and lysed with M-PER (Thermo Scientific) supplemented with protease inhibitor (Roche) and phosphatase inhibitors (Sigma–Aldrich). Cell lysates were separated using SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and were transferred to a PVDF membrane. The membranes were probed with the primary antibodies followed by a horseradish peroxidase (HRP)-conjugated secondary antibody. The bands were visualized using ECL Plus (GE Healthcare).

### 2.5. Radiosensitivity assay

Radiosensitivity was determined using a crystal violet assay, as described previously [11,12]. Briefly, cells treated with siRNA were exposed to γ-irradiation at room temperature using a Gammacell instrument (Gammacell 40 Exactor) capable of providing a dose rate of 1.04 Gy/min. After irradiation, the cells were cultured for 7 days, then fixed with glutaraldehyde and stained with crystal violet. The absorbance values of the extracted stain at 549 nm were measured, and cell survival was determined by normalization to a non-irradiated control.

### 2.6. Cell cycle analysis

The cell cycle of cells treated with siRNA and exposed to radiation was analyzed using the BD Cycletest™ Plus DNA Reagent Kit (BD Biosciences) and a FACS Calibur (BD Biosciences), according to the manufacturer's instructions.

### 2.7. Statistical analysis

All the data were presented as the mean ± standard deviation (SD). Results were considered statistically significant at $P < 0.05$. All the statistical analyses were performed using JMP 9 (SAS Institute).

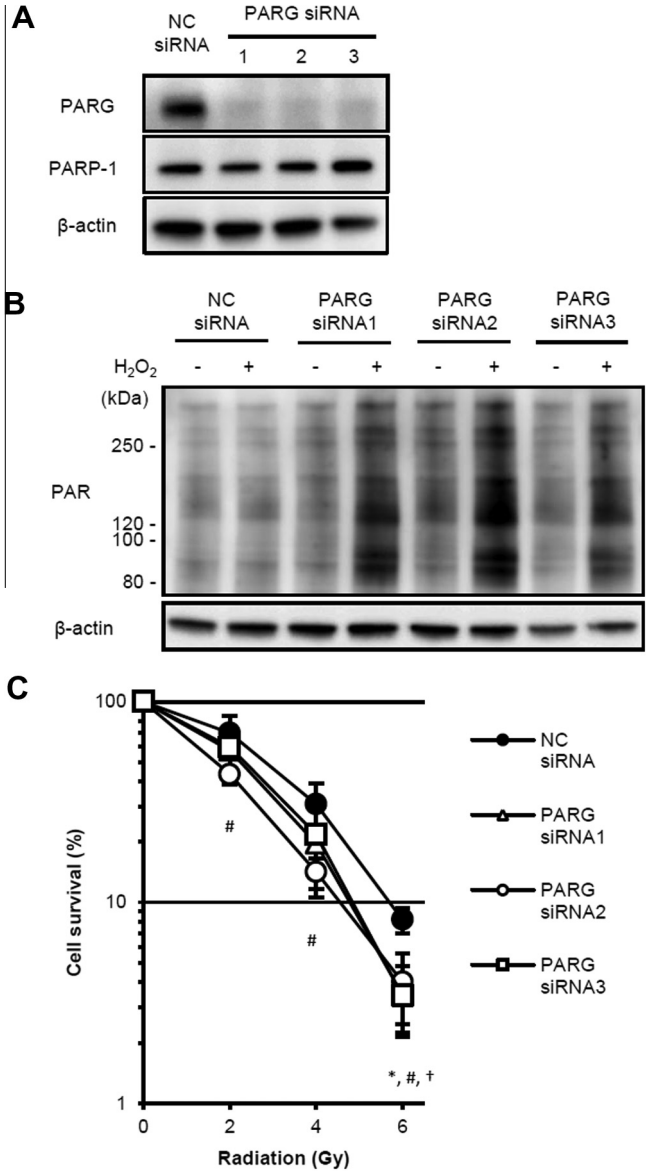


**Fig. 1.** Silencing of PARG enhanced the accumulation of PAR induced by $H_2O_2$ and the sensitivity to radiation in PC-14 cells. (A) PC-14 cells were transfected with NC or PARG siRNA. Cells were collected and lysed at 48 h after transfection. The expression levels of PARG and PARP-1 were examined using Western blotting. (B) Cells were exposed to 1 mM $H_2O_2$ for 10 min at 48 h after siRNA transfection. Poly ADP ribosylated proteins were detected using Western blotting with anti-PAR antibody. (C) Cells were irradiated with the indicated doses at 48 h after treatment with siRNA for NC or PARG. Survival was evaluated using crystal violet staining on day 7 after irradiation. The data show the mean ± SD of three independent experiments. *, #, †$P < 0.05$, cells treated with PARG siRNA1, siRNA2 or siRNA3, compared with NC siRNA-treated cells, respectively.

## 3. Results

We first examined the knock-down effect of three different siRNAs specific for PARG that target all the isoforms of PARG. All three siRNAs largely decreased the expression levels of PARG protein in PC-14, a lung cancer cell line (Fig. 1A). In contrast, the PARP-1 protein level was not changed by the siRNAs for PARG (Fig. 1A). Next, we investigated whether a functional defect could be achieved using siRNA for PARG. The accumulation of PAR was clearly observed only in PARG siRNA-treated cells when the cells were exposed to $H_2O_2$ for 10 min (Fig. 1B). The sensitivity to radiation of PC-14 treated with a negative control (NC) or PARG siRNA was assessed using a crystal violet assay on day 7 after irradiation. The cells treated with PARG siRNA showed a significantly increased radiosensitivity, compared with those of cells treated with NC siRNA (Fig. 1C).

Further evaluation of the radiosensitizing effect of PARG siRNA was conducted using 7 additional lung cancer cell lines. We chose PARG siRNA2 for use in the following experiments because similar results were obtained in PC-14 when all three PARG siRNAs were used. Using Western blotting, we confirmed that all 7 cell lines showed decreased levels of PARG protein at 48 h after treatment with the specific siRNA (Fig. 2A). A427, EBC-1, H2170 and SBC-5 displayed a significantly augmented radiosensitivity after PARG silencing (Fig. 2B). In contrast, the remaining 3 cell lines, H23, H520 and SBC-3, did not exhibit any augmentation in radiosensitivity, although PARG expression was similarly decreased (Fig. 2A and B).

We next focused on cell cycle after irradiation in NC or PARG siRNA-treated cells. The cell cycle was evaluated using 2 radiosensitizing cell lines, PC-14 and A427, and 1 non-radiosensitizing cell line, H520, using PARG knock-down. All 3 cell lines commonly exhibited radiation-induced G2/M arrest at 8–24 h after irradiation when treated with NC siRNA (Fig. 3A and B). On the other hand, when treated with PARG siRNA, irradiation-induced G2/M arrest was suppressed only in the PC-14 and A427 cell lines, but not the H520 cell line (Fig. 3A and B). This result suggested that the abrogation of cell cycle arrest was associated with radiosensitization through the silencing of PARG.

Finally, we examined the DNA damage checkpoint signals using Western blotting to clarify the mechanism underlying the dysregulation of the cell cycle after irradiation in PARG siRNA-treated cells. In PC-14 cells treated with NC siRNA, PAR was accumulated immediately after irradiation (10 min) but had decreased almost to base-line levels by 1 h after irradiation. In contrast, PAR continued to increase notably until 1 h after irradiation in PARG siRNA-treated PC-14 cells (Fig. 4A), and the accumulation of PAR occurred in a dose-dependent manner (Fig. 4B). PARG siRNA led to the abun-

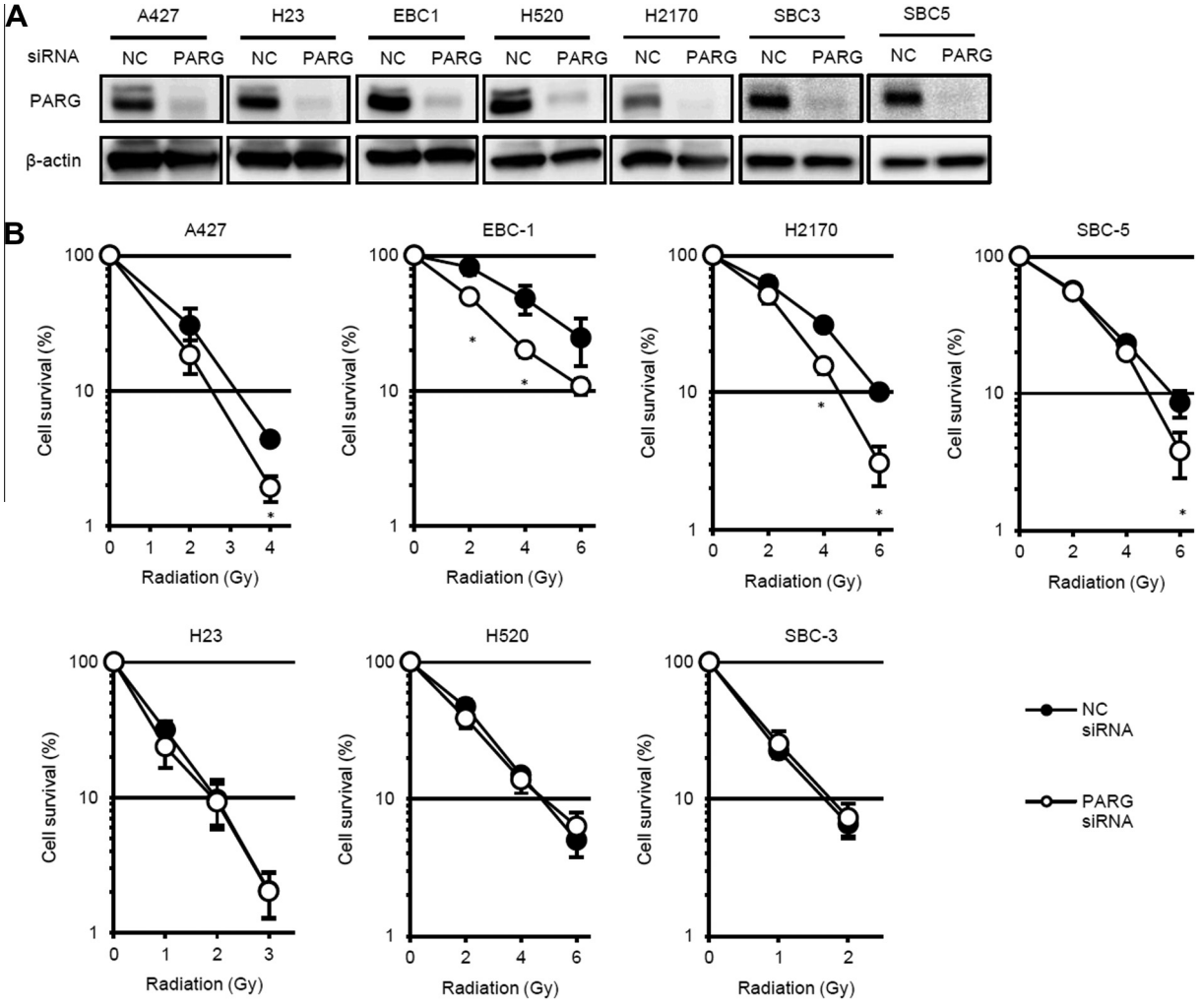


**Fig. 2.** Radiosensitization effect of PARG siRNA observed in multiple lung cancer cells. (A) Cells were treated with NC or PARG siRNA. Cells were collected and lysed at 48 h after transfection. The expression levels of PARG were examined using Western blotting. (B) Cells were irradiated with the indicated doses at 48 h after treatment with siRNA for NC or PARG. Survival was evaluated using crystal violet staining on day 7 after irradiation. The data show the mean ± SD of at least three independent experiments. $^*P < 0.05$, compared with NC siRNA-treated cells.

dant accumulation of PAR in A427 cells prior and post to irradiation (Fig. 4A). The phosphorylation of ATM, Chk1, and Chk2 was induced immediately after irradiation (10 min) in both cell lines treated with NC siRNA, whereas PARG siRNA-treated cells displayed decreased levels of induction, especially for ATM and Chk1 (Fig. 4A and B). Consistent with the cell cycle data, these results suggested that the silencing of PARG leads to the breakdown of the checkpoint mechanism, which may contribute to the sensitivity of the cell to radiation. Taken together, these results suggested that the silencing of PARG could be an attractive therapeutic strategy for lung cancer treatment when used in combination with radiotherapy.

## 4. Discussion

Until now, although PARG inhibition has been reported to contribute to an increased sensitivity to radiation, the mechanisms of action have been discussed in a cell type-dependent manner. For example, Ame et al. demonstrated that HeLa-derived PARG-deficient cells exhibited enhanced radiosensitivity through a delay in SSB and DSB repair and the enhanced induction of mitotic catastrophe [7]. In contact, Shirai et al. reported that the defective repair of DDB resulted in the induction of apoptosis in PARG-deficient mouse ES cells [8]. In the present study, we found that PARG siRNA led to the abrogation of radiation-induced G2/M arrest and checkpoint activation. This situation may have contributed to the sensitization of cells to the effects of radiation, since it occurred only in cells that exhibited a radiosensitizing effect. In contrast to previous reports, these cells did not show an increased sub-G1 or polyploidy population, which indicates apoptosis or a mitotic abnormality, respectively.

G2/M arrest is induced transiently to protect cells from DNA damage. The abrogation of the G2/M checkpoint leads to a decrease in repair activity for DNA damage [13]. In the present study, we demonstrated that PARG siRNA negatively affects the phosphorylation of ATM soon after radiation (Fig. 4A). ATM is a key enzyme for DNA damage checkpoints, and early activation is important for DNA break repair. Using a PARP inhibitor, Haince et al. demonstrated that the formation of PAR is involved in the recruitment

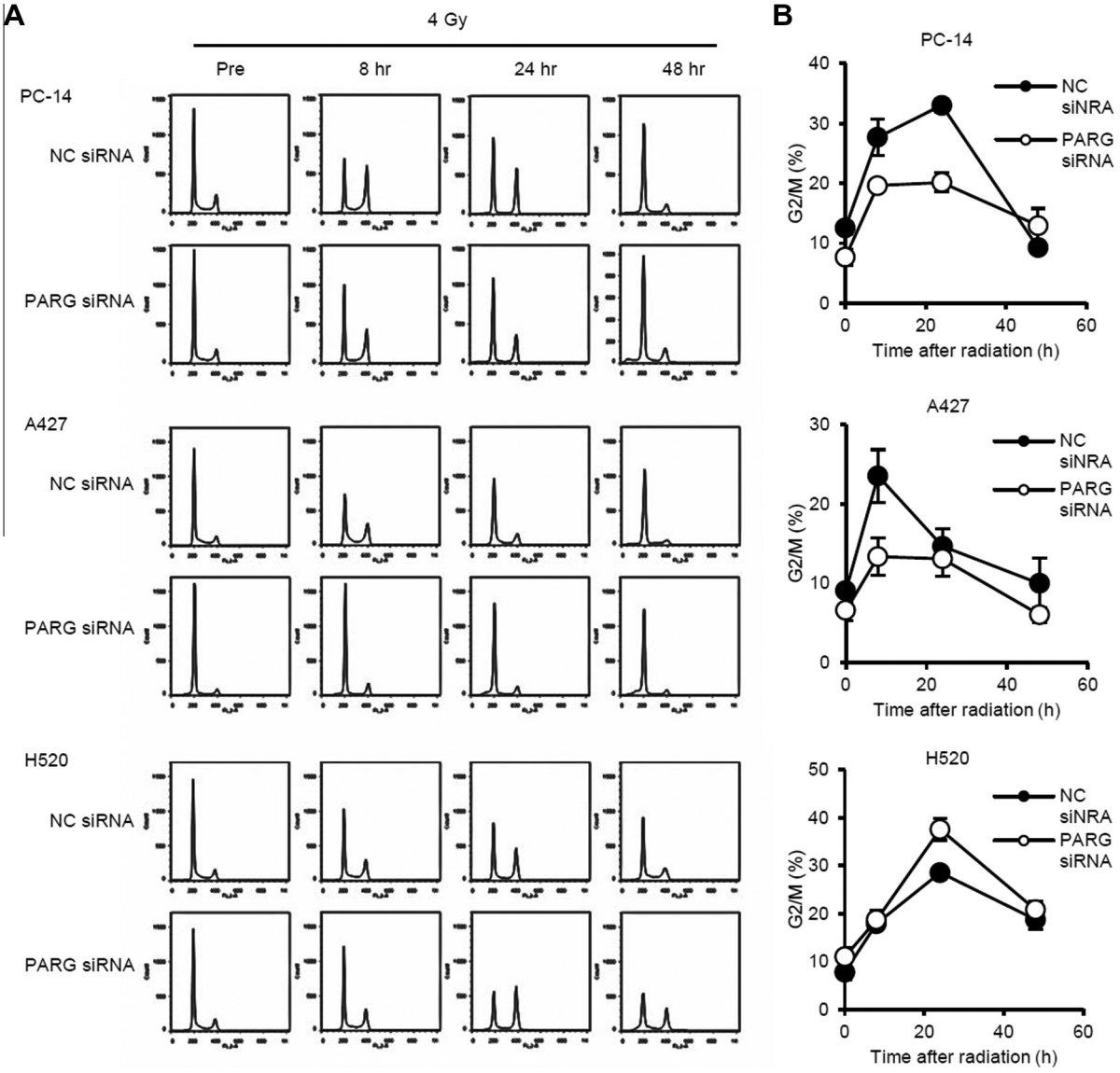


**Fig. 3.** Radiation-induced G2/M arrest was impaired by PARG siRNA. (A) Cells were irradiated with 4 Gy at 48 h after treatment with siRNA for NC or PARG. Cells were harvested at the indicated time after irradiation. For the cell-cycle analyses, the cells were analyzed using flow cytometry. The data are representative of three independent experiments. (B) The proportion of G2/M was calculated using FlowJo software. The data show the mean ± SD of three independent experiments.

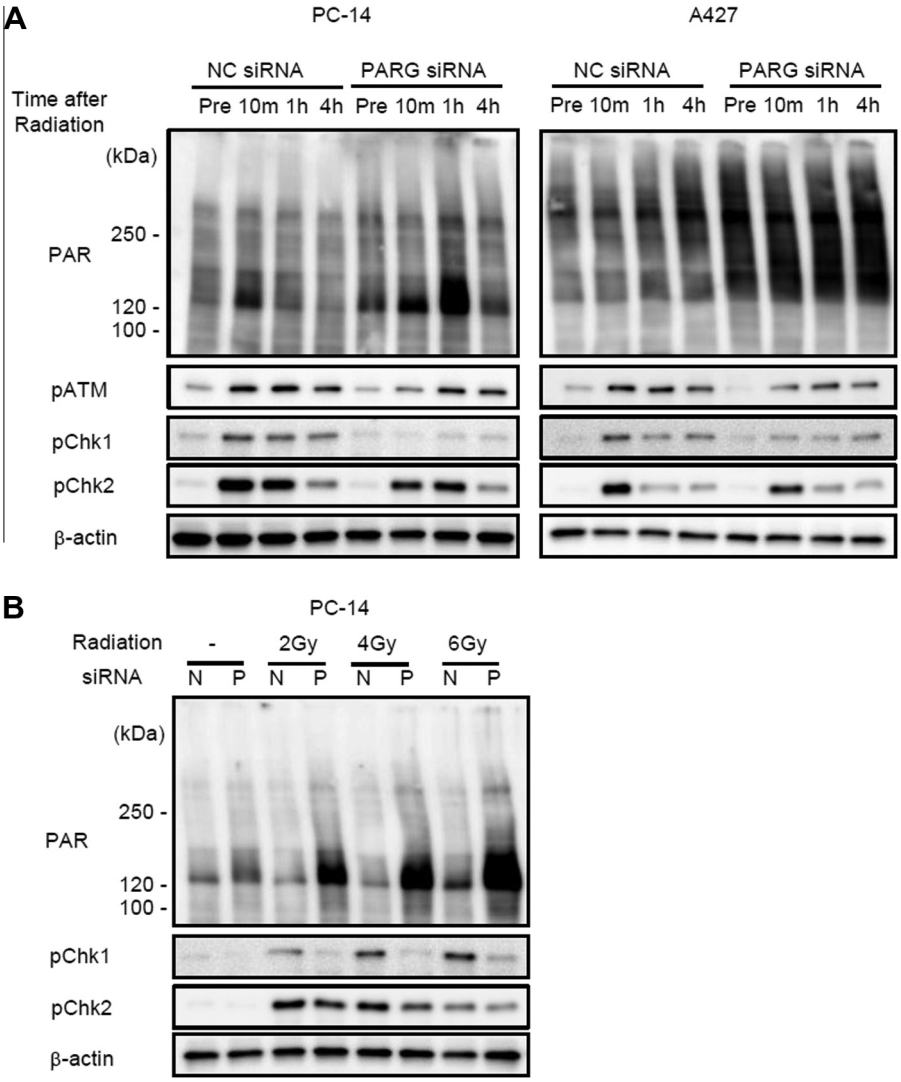


**Fig. 4.** Radiation-induced DNA-damage checkpoint signals were suppressed by PARG siRNA. (A) Cells were irradiated with 4 Gy at 48 h after treatment with siRNA for NC or PARG. Cells were harvested at the indicated times after irradiation. PAR and checkpoint proteins were evaluated using Western blotting. (B) Cells were irradiated with the indicated doses at 48 h after treatment with siRNA for NC (N) or PARG (P). Cells were harvested at 1 h after irradiation. PAR and checkpoint proteins were evaluated using Western blotting.

of ATM to DNA-damaged sites and ATM activation [14]. Our experiments showed the excessive and retained accumulation of PAR in PARG-deficient cells after irradiation (Fig. 4A and B). These observations may indicate that adequate PAR metabolism is crucial for ATM-initiated DNA damage signaling.

We also showed that the phosphorylation of checkpoint kinases, especially Chk1, was suppressed in PARG-deficient cells after irradiation (Fig. 4A and B). Chk1 and Chk2 were activated by ATM after DNA damage and induced cell cycle arrest. Chk1 is already considered to be a promising target for cancer treatment as a component in chemoradiotherapy [15]. A Chk1 inhibitor combined with radiation exerted augmented effects via a decrement in the pChk1 levels and the G2/M arrest induced by radiation [16,17], consistent with our results. Therefore, the suppression of Chk1 activation is considered to be a key step in the radiosensitizing effect induced by PARG inhibition.

In this study, 3 of the 8 cells that were studied did not display a radiosensitizing effect, even though PARG protein was successfully decreased in these cells as well as in cells that showed an increased sensitivity to radiation (Fig. 2A and B). The apparent difference in the efficacy of radiosensitization implies the presence of a predictive or resistant factor. The P53 mutation status was frequently reported to be involved in the radiosensitizing effect of targeted therapy [18,19]. However, the P53 mutation status was not associated with a radiosensitizing effect in our study (data not shown). Predictive factors should be investigated in the future to allow individualized medicine involving PARG inhibitors.

In conclusion, we have demonstrated that the knockdown of PARG using siRNA resulted in the enhanced sensitivity of multiple lung cancer cell lines to radiation. Our results provide a rationale for PARG inhibition combined with radiation in patients with lung cancer.

## Acknowledgment

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## References

[1] M. Rouleau, A. Patel, M.J. Hendzel, S.H. Kaufmann, G.G. Poirier, PARP inhibition: PARP1 and beyond, Nat. Rev. Cancer 10 (2010) 293–301.

[2] S.F. El-Khamisy, M. Masutani, H. Suzuki, K.W. Caldecott, A requirement for PARP-1 for the assembly or stability of XRCC1 nuclear foci at sites of oxidative DNA damage, Nucleic Acids Res. 31 (2003) 5526–5533.
[3] G. Brochu, C. Duchaine, L. Thibeault, J. Lagueux, G.M. Shah, G.G. Poirier, Mode of action of poly(ADP-ribose) glycohydrolase, Biochim. Biophys. Acta 1219 (1994) 342–350.
[4] W. Lin, J.C. Ame, N. Aboul-Ela, E.L. Jacobson, M.K. Jacobson, Isolation and characterization of the cDNA encoding bovine poly(ADP-ribose) glycohydrolase, J. Biol. Chem. 272 (1997) 11895–11901.
[5] R.G. Meyer, M.L. Meyer-Ficca, C.J. Whatcott, E.L. Jacobson, M.K. Jacobson, Two small enzyme isoforms mediate mammalian mitochondrial poly(ADP-ribose) glycohydrolase (PARG) activity, Exp. Cell Res. 313 (2007) 2920–2936.
[6] O. Mortusewicz, E. Fouquerel, J.C. Ame, H. Leonhardt, V. Schreiber, PARG is recruited to DNA damage sites through poly(ADP-ribose)- and PCNA-dependent mechanisms, Nucleic Acids Res. 39 (2011) 5045–5056.
[7] J.C. Ame, E. Fouquerel, L.R. Gauthier, D. Biard, F.D. Boussin, F. Dantzer, G. de Murcia, V. Schreiber, Radiation-induced mitotic catastrophe in PARG-deficient cells, J. Cell Sci. 122 (2009) 1990–2002.
[8] H. Shirai, H. Fujimori, A. Gunji, D. Maeda, T. Hirai, A.R. Poetsch, H. Harada, T. Yoshida, K. Sasai, R. Okayasu, M. Masutani, Parg deficiency confers radiosensitization through enhanced cell death in mouse ES cells exposed to various forms of ionizing radiation, Biochem. Biophys. Res. Commun. 435 (2013) 100–106.
[9] J.K. Salama, E.E. Vokes, New radiotherapy and chemoradiotherapy approaches for non-small-cell lung cancer, J. Clin. Oncol. 31 (2013) 1029–1038.
[10] G.M. Videtic, The role of radiation therapy in small cell lung cancer, Curr. Oncol. Rep. 15 (2013) 405–410.
[11] F. Essmann, I.H. Engels, G. Totzke, K. Schulze-Osthoff, R.U. Janicke, Apoptosis resistance of MCF-7 breast carcinoma cells to ionizing radiation is independent of p53 and cell cycle control but caused by the lack of caspase-3 and a caffeine-inhibitable event, Cancer Res. 64 (2004) 7065–7072.
[12] K. Toth, V. Tarakanova, K. Doronin, P. Ward, M. Kuppuswamy, J.E. Locke, J.E. Dawson, H.J. Kim, W.S. Wold, Radiation increases the activity of oncolytic adenovirus cancer gene therapy vectors that overexpress the ADP (E3-11.6K) protein, Cancer Gene Ther. 10 (2003) 193–200.
[13] M.A. Morgan, L.A. Parsels, L. Zhao, J.D. Parsels, M.A. Davis, M.C. Hassan, S. Arumugarajah, L. Hylander-Gans, D. Morosini, D.M. Simeone, C.E. Canman, D.P. Normolle, S.D. Zabludoff, J. Maybaum, T.S. Lawrence, Mechanism of radiosensitization by the Chk1/2 inhibitor AZD7762 involves abrogation of the G2 checkpoint and inhibition of homologous recombinational DNA repair, Cancer Res. 70 (2010) 4972–4981.
[14] J.F. Haince, S. Kozlov, V.L. Dawson, T.M. Dawson, M.J. Hendzel, M.F. Lavin, G.G. Poirier, Ataxia telangiectasia mutated (ATM) signaling network is modulated by a novel poly(ADP-ribose)-dependent pathway in the early response to DNA-damaging agents, J. Biol. Chem. 282 (2007) 16441–16453.
[15] M. Maugeri-Sacca, M. Bartucci, R. De Maria, Checkpoint kinase 1 inhibitors for potentiating systemic anticancer therapy, Cancer Treat. Rev. 39 (2013) 525–533.
[16] Y. Tao, C. Leteur, C. Yang, P. Zhang, M. Castedo, A. Pierre, R.M. Golsteyn, J. Bourhis, G. Kroemer, E. Deutsch, Radiosensitization by Chir-124, a selective CHK1 inhibitor: effects of p53 and cell cycle checkpoints, Cell Cycle 8 (2009) 1196–1205.
[17] G.R. Borst, M. McLaughlin, J.N. Kyula, S. Neijenhuis, A. Khan, J. Good, S. Zaidi, N.G. Powell, P. Meier, I. Collins, M.D. Garrett, M. Verheij, K.J. Harrington, Targeted radiosensitization by the Chk1 inhibitor SAR-020106, Int. J. Radiat. Oncol. Biol. Phys. 85 (2013) 1110–1118.
[18] L. Biddlestone-Thorpe, M. Sajjad, E. Rosenberg, J.M. Beckta, N.C. Valerie, M. Tokarz, B.R. Adams, A.F. Wagner, A. Khalil, D. Gilfor, S.E. Golding, S. Deb, D.G. Temesi, A. Lau, M.J. O'Connor, K.S. Choe, L.F. Parada, S.K. Lim, N.D. Mukhopadhyay, K. Valerie, ATM kinase inhibition preferentially sensitizes p53-mutant glioma to ionizing radiation, Clin. Cancer Res. 19 (2013) 3189–3200.
[19] S. Vance, E. Liu, L. Zhao, J.D. Parsels, L.A. Parsels, J.L. Brown, J. Maybaum, T.S. Lawrence, M.A. Morgan, Selective radiosensitization of p53 mutant pancreatic cancer cells by combined inhibition of Chk1 and PARP1, Cell Cycle 10 (2011) 4321–4329.