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ABSTRACT

BACKGROUND: Triple-negative breast cancer (TNBC) exhibits poor prognosis due to the lack of targets for hormonal or antibody-based therapies, thereby leading to limited success in the treatment of this cancer subtype. Poly (ADP-ribose) polymerase 1 (PARP1) is a critical factor for DNA repair, and using PARP inhibitor (PARPi) is one of the promising treatments for BRCA-mutated (BRCA mut) tumors where homologous recombination repair is impaired due to BRCA1 mutation. Carbon ion (C-ion) radiotherapy effectively induces DNA damages in cancer cells. Thus, the combination of C-ion radiation with PARPi would be an attractive treatment for BRCA mut TNBC, wherein DNA repair systems can be severely impaired on account of the BRCA mutation. Till date, the effectiveness of C-ion radiation with PARPi in BRCA mut TNBC cell killing remains unknown.

PURPOSE: Triple-negative breast cancer cell lines carrying either wild type BRCA1, BRCA wt, (MDA-MB-231), or the BRCA1 mutation (HCC1937) were used, and the effectiveness of PARPi, olaparib, combined with C-ion beam or the conventional radiation, or X-ray, on TNBC cell killing were investigated.

METHODS: First, effective concentrations of olaparib for BRCA mut (HCC1937) cell killing were identified. Using these concentrations of olaparib, we then investigated their radio-sensitizing effects by examining the surviving fraction of MDA-MB-231 and HCC1937 upon X-ray or C-ion irradiation. In addition, the number of γH2AX (DSB marker) positive cells as well as their expression levels were determined by immunohistochemistry, and results were compared between X-ray irradiated or C-ion irradiated cells. Furthermore, PARP activities in these cells were also observed by performing immunohistochemistry staining for poly (ADP-ribose) polymer (marker for PARP activity), and their expression differences were determined.

RESULTS: Treatment of cells with 25 nM olaparib enhanced radio-sensitivity of X-ray irradiated HCC1937, whereas lower dose (5 nM) olaparib showed drastic effects on increasing radio-sensitivity of C-ion irradiated HCC1937. Similar effect was not observed in MDA-MB-231, not possessing the BRCA1 mutation. Results of immunohistochemistry showed that X-ray or C-ion irradiation induced similar number of γH2AX-positive HCC1937 cells, but these induction levels were higher in C-ion irradiated HCC1937 with increased PARP activity compared to that of X-ray irradiated HCC1937. Elevated induction of DSB in C-ion irradiated HCC937 may fully activate DSB repair pathways leading to downstream activation of PARP, subsequently enhancing the effectiveness of PARPi, olaparib, with lower doses of olaparib exerting noticeable effects in cell killing of C-ion irradiated HCC1937.

CONCLUSIONS: From this study, we demonstrate that C-ion irradiation can exert significant DSB in BRCA mut TNBC, HCC1937, with high PARP activation. Thus, PARPi, olaparib, would be a promising candidate as a radio-sensitizer for BRCA mut TNBC treatment, especially for C-ion radiotherapy.

KEYWORDS: Triple-negative breast cancer, PARP, C-ion beam, olaparib, radio-sensitizer, chemoradiotherapy

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Introduction

Breast cancer (BC) is the most commonly occurring cancer in women. The number of new cases per year is more than 93,000 in Japan, and 14,839 women succumbed to this disease in 2019.[1] Tumor stage, tumor grade, hormone receptor status, and HER2 status are commonly used to make prognosis and treatment decisions for BC patients,[2] and major subtypes of BC are approximated by the joint expression of the hormone receptors, estrogen receptor (ER) and progesterone receptor (PR), and HER2 amplification status. Among all, the subtypes of BC, triple-negative breast cancer (TNBC), which shows approximately 15% to 20% of all BC, is recognized as one of the most

difficult BC to treat.[3] Owing to the lack expression of ER, PR, and no amplification of HER2 gene, TNBC does not respond to hormonal or antibody-based targeted therapies, which causes limited success in treatment and management of this disease.

Genomic instability is one of the enabling characteristics of tumor development.[4] Since DNA repair genes play a significant role in maintaining genomic integrity, defects in these DNA repair genes are often found in tumor. In fact, germline mutations in the BRCA1 gene, which plays a key role in DNA double-strand break (DSB) repair via homologous recombination (HR),[5] are associated with TNBC, and approximately 70% of BRCA1-mutated (BRCA mut) BC are diagnosed as TNBC.[6] Studies have reported that Poly (ADP-ribose) polymerase (PARP) inhibition is a promising approach for the targeted treatment of BRCA-deficient tumors.[7] Poly (ADP-ribose) polymerase 1 is a critical factor for several DNA repair mechanisms such as nucleotide excision repair (NER) and base excision repair system (BER) for DNA single-strand breaks (SSBs), and non-homologous end-joining system (NHEJ) for DNA double-strand breaks (DSBs).[8-10] Poly (ADP-ribose) polymerase 1 senses DNA strand breaks and transfers ADP-ribose units from NAD+ onto adjacent nuclear proteins (Poly(ADP-ribosyl)ation of proteins) that recruit repairing enzymes to the site of DNA repair.[7,9] The collapse in such DNA repair machineries using PARP inhibitor (PARPi) can lead to severe DNA damages via stalling of replication forks and generation of irreparable DNA DSBs.[11] It is known that such DSBs can only be repaired by HR that involves factors such as BRCA1/2,[12] thus, PARPi can induce severe cytotoxicity in BRCA mut tumors.[7,13] Proof-of-principle, studies of olaparib, a potent oral PARPi, have shown monotherapy activity and acceptable toxicity in patients with ovarian or BC who have a germline BRCA1 or BRCA2 mutation,[10,14] and olaparib has recently been approved by the US FDA and the European Commission for use as an anti-cancer drug for BRCA-mutated ovarian cancers.[15]

The National Institutes for Quantum and Radiological Science and Technology (QST) (formerly known as The National Institute of Radiological Sciences, NIRS) in Japan, started their first clinical trial for cancer treatment with carbon-ion (C-ion) beam in 1994. Among all high linear energy transfer (LET) radiations, C-ion beam is becoming increasingly popular for the treatment of malignant tumors because of its high-dose local distribution in the body,[16] achieving 90% or higher 5-year local control in some cases and 90% or higher 5-year patient survival.[17] We have also shown the merit of C-ion radiotherapy (RT) for BC in a phase-I clinical trial that was initiated in 2013.[18,19] However, some malignant tumors exhibit resistance to RT, and hence, candidates for use as radiosensitizers have been investigated in many studies.[20] DNA repair in cancer cells constitutes a major factor responsible for tumor resistance to RT. Therefore, inhibiting DNA repair in tumor cells by interfering with the functioning of DNA repair enzymes such as PARP1 is a rational therapeutic strategy to enhance the effects of radiation and could potentially develop into a new treatment strategy for TNBC. In fact, several studies have shown the effectiveness of PARPi as a radiosensitizer for X-ray irradiated cancer cells including TNBC cell lines.[21-23] Such effectiveness was especially noticeable in BRCA mut cells with high induction of DSBs and enhanced apoptosis.[21,22] In addition, Loap et al recently have started the RADIOPARP phase-1 trial to investigate the dose-limiting toxicities (DLTs) and the maximum tolerated dose of PARPi combined with locoregional radiation therapy of TNBC.[24] In another report, Hirai et al have demonstrated in an *in vitro* study that the PARPi can be an effective radiosensitizer for C-ion radiation in human pancreatic cancer cells,[25] but so far, effective radiosensitizers for C-ion radiation therapy for TNBC have not been reported. Since irradiation of tumors with C-ion radiation has advantages over the use of conventional photons with enhanced biological effects due to higher LET; C-ion beam can induce 2- to 3-fold greater cytotoxic effects in cancer cells, including severe DNA damages.[26] Thus, the combination of C-ion beam with PARPi would be an attractive treatment especially for BRCA mut TNBC, where DNA repair systems can be severely defective due to the BRCA mutation.

In this study, we used TNBC cell lines carrying either wild type (BRCA wt) or the mutant BRCA1 (BRCA mut) and identified the effectiveness of the combination of PARPi, olaparib, and C-ion beam in TNBC cell killing especially in BRCA-mutated TNBC cells.

Materials and Methods

Cells and reagents

Two human TNBC cell lines, MDA-MB-231 (BRCA wt) and HCC1937 (BRCA mut) were purchased from ATCC (Manassas, VA, USA). Both cell lines were cultured in RPMI 1640 (Nacalai, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (HyClone, UT, USA), and 1% penicillin/streptomycin (PS) (Gibco, MD, USA). Poly (ADP-ribose) polymerase inhibitor, olaparib, was purchased from ChemScene (NJ, USA) and used for this study at a concentration range of 5 nM to 1000 nM.

Irradiation

Cells were irradiated with C-ion beams accelerated by HIMAC at NIRS, QST. The initial energy of C-ion beams was 290 MeV/u, and the LET value was 80 keV/μm; a mono-energetic beam with a narrow Bragg Peak was applied at a depth of 10 cm, and cells were irradiated with 0, 0.5, 1, 1.5, or 2 Gy. For a comparison with the C-ion beams, 200 kV X-rays with 0, 1, 2, 3, or 4 Gy were used. X-rays were produced by PANTAC HF320-S X-ray generator (Shimadzu, Kyoto, Japan) at 200 kV, and 20 mA, and filtered with 0.5 mm Al and 0.5 mm Cu. All irradiations were carried out at a dose rate of approximately 1 Gy/min at room temperature. Cells were cultured on Falcon T25 flask (BD Falcon, NJ, USA) for 2 to 3 days before irradiation, and cell cultures at about 50% to 60% confluence were irradiated.

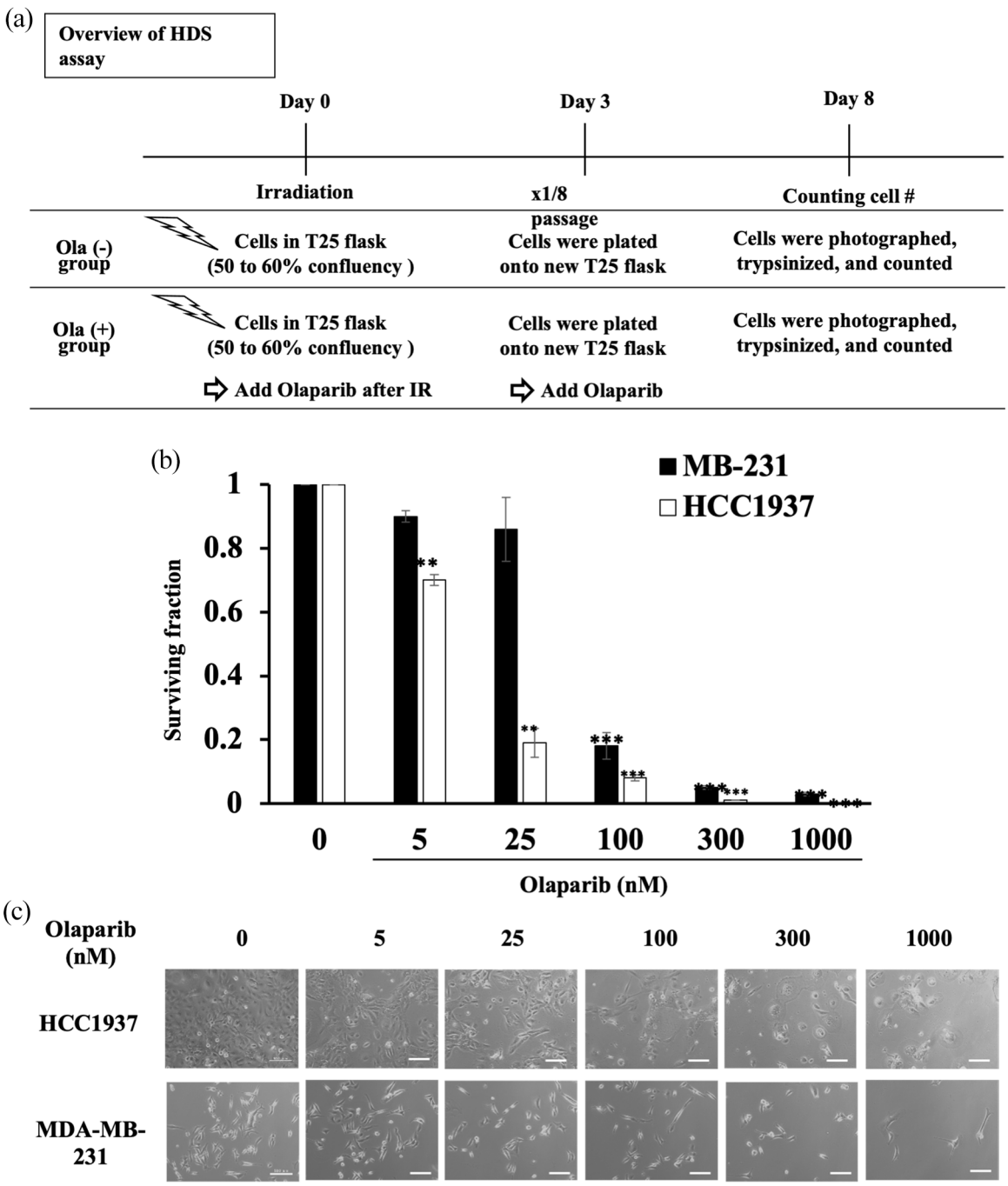


Figure 1. Defining the effective concentration of olaparib for BRCA mut cell killing: (A) protocol for HDS assay is summarized, (B) surviving fractions of breast cancer cell lines, HCC1937 and MDA-MB-231, upon treatment with various concentrations, 5-1000 nM, of olaparib, were determined using HDS assay, and were shown in graph. Data were normalized to control and shown as mean ± SD of triplicate samples. $^{**}P < .05$, $^{***}P < .001$. For the statistical analysis, unpaired Student's *t*-test was performed using GraphPad Prism 8. (C) Representative images of HCC1937 and MDA-MB-231 cells treated with olaparib are shown. Scale bar, 50 μm. BRCA mut indicates BRCA1 mutation; HDS, high-density survival; SD, standard deviation.

High-density survival assay (HDS)

The HDS assay was carried out as described by Karasawa et al, with some modifications.[27] Briefly, cells with about 50% to 60% confluence were irradiated, and the cells were kept in culture for an additional 3 days. Cells of each flask were then trypsinized and ×1/8 of cells for MDA-MB-231 and HCC1937 were plated onto new T25 flasks and subcultured further for 5 days. Eight days after exposure to radiation, cells were photographed with bright field microscope (Keyence, Osaka, Japan). Cells were then trypsinized, and the number of cells was counted with a hematocytometer. Outline of the experimental procedure for HDS assay after irradiation with olaparib treatment was summarized in Figure 1A.

Survival curves were fitted to the experimental data by regression analysis using the following linear quadratic equation[28]:

$$SF = \exp(-\alpha D - \beta D^2)$$

where SF is the surviving fraction and D is radiation dose (Gy).

Immunofluorescence labeling and image acquisition

Immunofluorescence labeling and image acquisition were performed as described previously, with some modifications.[29] Briefly, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; Nissui Pharmaceutical Co., Ltd.; Tokyo, Japan) for 15 min, and given three washes with PBS. Cells were then blocked with PBS containing 5% fetal calf serum and 0.3% Triton ×100, followed by incubation with primary antibody for 30 min at room temperature. The primary antibodies against phospho-Histone H2A.X at Ser139 (20E3) (Cell Signaling Technology., MA, USA), γH2AX, a marker for DNA damage,[30] and poly (ADP-ribose) polymer (Tulip BioLabs, PA, USA), PAR polymer, a marker of PARP activity,[31] were suspended in PBS containing 1% FCS and 0.3% Triton ×100 at 1:100. Dilution, and used for the assay. Cells were then treated with AlexaFluor 488- or AlexaFluor 555-labeled anti-mouse IgG or anti-rabbit IgG secondary antibodies (Invitrogen, Carlsbad, USA) for 30 min at room temperature. The slides were mounted with ProLong Gold Antifade Reagent containing the nuclear counterstain DAPI (Invitrogen). Fluorescent signal was visualized and photographed with a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan) using a 20× Plan fluorescence lens (N.A 0.45) with BZ filters for GFP-B, TRTIC, and DAPI. Representative images were uniformly processed in Adobe Photoshop using the brightness and contrast tools.

Counting of γH2AX+ PAR+ or γH2AX+ PAR– cells

Immunofluorescence-labelled cells were used to count the number of γH2AX and/or PAR-polymer-positive cells. DAPI images were first used to count the number of cell nuclei per image, representing the total number of cells per image. Next, the anti-γH2AX or anti-PAR polymer antibody-stained cells were counted, and the number of γH2AX and PAR-polymer-positive cells (γH2AX+ PAR+), and γH2AX+ positive, PAR-polymer-negative cells (γH2AX+ PAR–) were quantified. The ratio of γH2AX+ PAR+ or γH2AX+ PAR– within total number of cells was plotted in graph. The number of cells counted ranged from 9 to 217 per group.

Counting of γH2AX ^{high} or γH2AX ^{low} cells

Image J software program was used to count γH2AX ^{high} or γH2AX ^{low} expressing cells. First, the DAPI images were used to count the number of cell nuclei per image, representing the total number of cells per image. Next, anti-γH2AX antibody-stained images were converted into 8-bit grayscale images, and thresholding tool of the Image J software program, was used to separate the cells with low or high accumulation of γH2AX, by separating the pixels that fell within a desired range of intensity values from those that did not. The criteria used for the γH2AX ^{high} or γH2AX ^{low} was as follows: the cells in which the distribution of pixel intensity below 240 was classified as total number of γH2AX expressing cells (γH2AX ^{high} + γH2AX ^{low}), and the cells with the pixel intensity ⩾235 was classified as γH2AX ^{high}. For counting γH2AX ^{low} cells, the number of γH2AX ^{high} cells was subtracted from the number of γH2AX ^{high} + γH2AX ^{low}. The ratio of γH2AX ^{high} or γH2AX ^{low} within total number of cells was plotted in graph. The number of cells counted ranged from 9 to 217 per group.

Statistical analysis

All results are expressed as the mean ± SD. Statistical analyses, unpaired Student's *t*-test or two-way ANOVA, were performed using GraphPad Prism 8 (GraphPad Software Inc., California San Diego, USA). *P* value of $<.05$ was considered significant.

Results

Defining the effective concentration of olaparib for BRCA mut cell killing

To decide the effective concentration of PARPi, olaparib, especially for BRCA mut cell killing, two TNBC cell lines, HCC1937 (BRCA mut cell line) and MDA-MB-231 (BRCA wt cell line), were used in this study. HDS assay (Figure 1A) showed that the survival rate of HCC1937 was significantly lower than that of MDA-MB-231 at any concentration (5, 25, 100, 300, or 1000 nM olaparib) (Figure 1B and C). The difference in cell killing between HCC1937 versus MDA-MB-231 was the most apparent at 25 nM olaparib, 79% reduction in HCC1937 and 24% reduction in MDA-MB-231 were observed in cells treated with 25 nM olaparib. Thus, 25 nM olaparib would be the most effective concentration for selective killing of HCC1937 (BRCA mut) cell compared to killing of MDA-MB-231 (BRCA wt), and this would be appropriate concentration to use further in this study. In addition, we observed that 100, 300, or 1000 nM olaparib was too high to use as a PARPi because the survival rate was drastically reduced even in MDA-MB-231 that were not inherently highly sensitive (BRCA wt) to PARPi (Figure 1B and C).

The main objective of this study was to examine the effects of a combination of olaparib and radiation on BRCA mut cell killing, preferentially by using olaparib at concentrations wherein olaparib by itself induced low, if any, cytotoxic effects. Therefore, we decided to use two olaparib concentrations, the moderately effective dose, 5 nM, and the most effective dose, 25 nM, and examine the effectiveness of olaparib as a radiosensitizer in combination with X-ray or C-ion radiation.

Olaparib-enhanced radio-sensitivity of HCC1937 to X-ray irradiation

First, the combined effect of olaparib with X-ray irradiation was determined. X-ray irradiation by itself reduced survival rate of both cell lines, HCC1937 and MDA-MB-231, in a dose-dependent manner (Upper panel of Figure 2A and B, respectively). Treatment of cells with 5 nM olaparib, the moderate effective dose, tended to increase the radio-sensitivity (tended to reduce the survival fraction) of only the X-ray irradiated HCC1937 (BRCA mut) and not MDA-MB-231 (Middle panel of Figure 2A and C). In contrast, the most effective dose of olaparib, 25 nM, showed significantly higher effectiveness on upregulating radio-sensitivity of 1 Gy, or 2-Gy X-ray irradiated HCC1937 cells compared to that of no olaparib treatment group (lower vs upper panel of Figure 2A and C), while no such significant increase in radio-sensitivity was observed in X-ray irradiated MDA-MB-231 with 5 or 25 nM olaparib (lower vs upper panel of Figure 2B and D). These data suggest that the administration of olaparib at an appropriate concentration, 25 nM, to HCC1937 (BRCA mut) produces a sensitizing effect to X-ray irradiation. In contrast, such effects were not obtained in MDA-MB-231, indicating that olaparib could selectively sensitize BRCA1-mutated cancer cells to X-ray irradiation.

Low-dose of olaparib was effective in increasing radio-sensitivity of HCC1937 irradiated with C-ion beam

Next, we examined a sensitizing effect of olaparib in C-ion irradiated cells. As observed in the case of X-rays, C-ion beam itself also reduced the survival fraction of both HCC1937 and MDA-MB-231 cell lines in a dose-dependent manner (upper panel of Figure 3A and B, respectively). Importantly, in contrast to X-ray radiation, 5 nM olaparib, the moderate effective dose, showed drastic effect on cell killing of 0.5 Gy or 1-Gy-irradiated HCC1937 compared to the no olaparib treatment group, indicating that this moderate dose can elevate radio-sensitivity of HCC1937 to 0.5 Gy or 1 Gy-C-ion irradiation (middle vs upper panel of Figure 3A and C). In addition, 25 nM olaparib also showed similar significant effects on reducing surviving fraction of 0.5 Gy or 1 Gy-C-ion irradiated HCC1937 (lower vs upper panel of Figure 3A and C). In the case of MDA-MB-231 (BRCA wt), there was no significant difference in the survival rate at any olaparib concentration (Figure 3B and D), indicating that olaparib had less effects on enhancing their radio-sensitivity.

Since lower dose (5 nM) of olaparib, had a significant effect in increasing the sensitivity of HCC1937 to C-ion radiation compared to X-ray radiation, olaparib was suggested as an effective radio-sensitizer for BRCA mut TNBC, especially in combination with C-ion radiation.

X-ray or C-ion radiation induced similar number of γH2AX-positive HCC1937 cells with higher induction levels in C-ion irradiated HCC1937 with greater effectiveness of olaparib

Our results thus far showed that the administration of olaparib after C-ion irradiation had a noticeable sensitizing effect on HCC1937 (BRCA mut) with lower concentration of olaparib, 5 nM, compared to X-ray irradiation. Thus, olaparib was suggested as an effective radio-sensitizer specially for C-ion radiation. It is well known that C-ion beam can cause about 2-fold greater cytotoxicity than X-ray radiation in cancer cells.[26,27] Consistently, we observed that 2 Gy X-ray radiation or 1 Gy C-ion radiation (half the radiation dose compared to the dose of X-ray), exerted similar effects on HCC1937 surviving fraction, which were 41 ± 26% for 2 Gy X-ray irradiated and 41 ± 23% for 1 Gy C-ion irradiated HCC1937, respectively (Figures 2C and 3C), indicated that C-ion beam exerted about 2-fold higher cytotoxicity in HCC1937 cells. Thus, we hypothesized that 2 Gy X-ray radiation and 1 Gy C-ion radiation can also give similar cytotoxic effects via comparable induction of DNA damage. To clarify this, we irradiated HCC1937 with 2 Gy X-ray or 1 Gy C-ion beam, and examined the number of γH2AX (a marker of DNA DSB) induced cells. As expected, the number of γH2AX positive cells was comparably increased in both groups; 80 ± 9% or 96 ± 6% cells within total cell number showed γH2AX induction upon 2 Gy X-ray or 1 Gy C-ion irradiation, respectively (upper panel of green staining in Figure 4A, and X-ray with Ola(–) versus C-ion with Ola(–) in Figure 4B). Poly (ADP-ribose) polymerase 1 is well-known as a significant factor for SSB repair machinery, and PARPi, olaparib, can trap PARP onto DNA at SSBs, leading to collapse in replication forks and subsequent induction of DNA DSBs.[11]

Once DSB are formed, DSB repair system such as HR and NHEJ are known to be activated.[32] Since HCC1937 is an HR-deficient tumor (BRCA mutant), thus NHEJ would be the alternative machinery to repair the DSB in these cells. Poly (ADP-ribose) polymerase is thought to play a role in NHEJ in 2 Gy X-ray or 1 Gy C-ion irradiation in HCC1937, and thus, we further examined the level of poly (ADP-ribose) polymer, a marker for PARP1 activity in these cells (red staining of upper panel in Figure 4A). Surprisingly, the number of polymer positive cells were significantly increased further upon 1 Gy C-ion irradiation compared to 2 Gy X-ray irradiated HCC1937, at 89 ± 19% and 64 ± 12%, respectively (Figure 4B: black bar of C-ion with Ola(–) vs Black bar of X ray with Ola(–), $P = .048$), indicated that DNA repair system with PARP was highly activated specially in C-ion irradiated HCC1937. Furthermore, moderate dose (5 nM) of olaparib, when combined with 1 Gy C-ion irradiation, drastically reduced the number of polymer positive cells (19 ± 19%), an effect that is not observed in combination with 2Gy X-ray irradiation (64 ± 9%) (red staining of

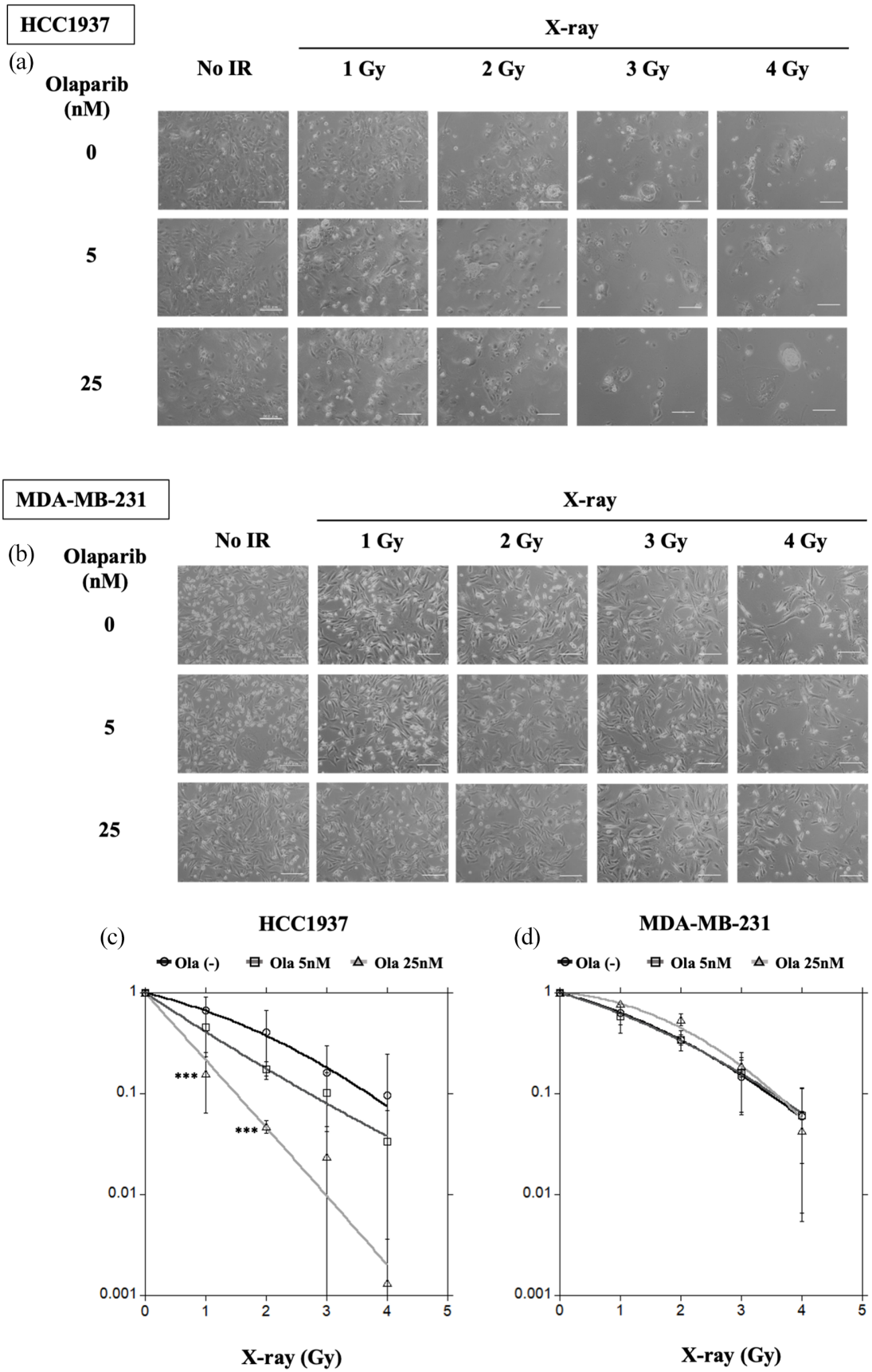


Figure 2. Olaparib increased radio-sensitivity of X-ray irradiated HCC1937 but not MDA-MB-231. Representative images of HCC1937 (A) and MDA-MB-231 (B) treated with X-ray irradiation and olaparib, and corresponding HDS survival curves of HCC1937 (C) and MDA-MB-231 (D) were shown in graph, respectively. Scale bar, 50 μm. Data in the graph show the mean ± SD of triplicate samples. ***$P < .001$. For the statistical analysis, two-way ANOVA was performed using GraphPad Prism 8. ANOVA indicates analysis of variance; HDS, high-density survival; SD, standard deviation.

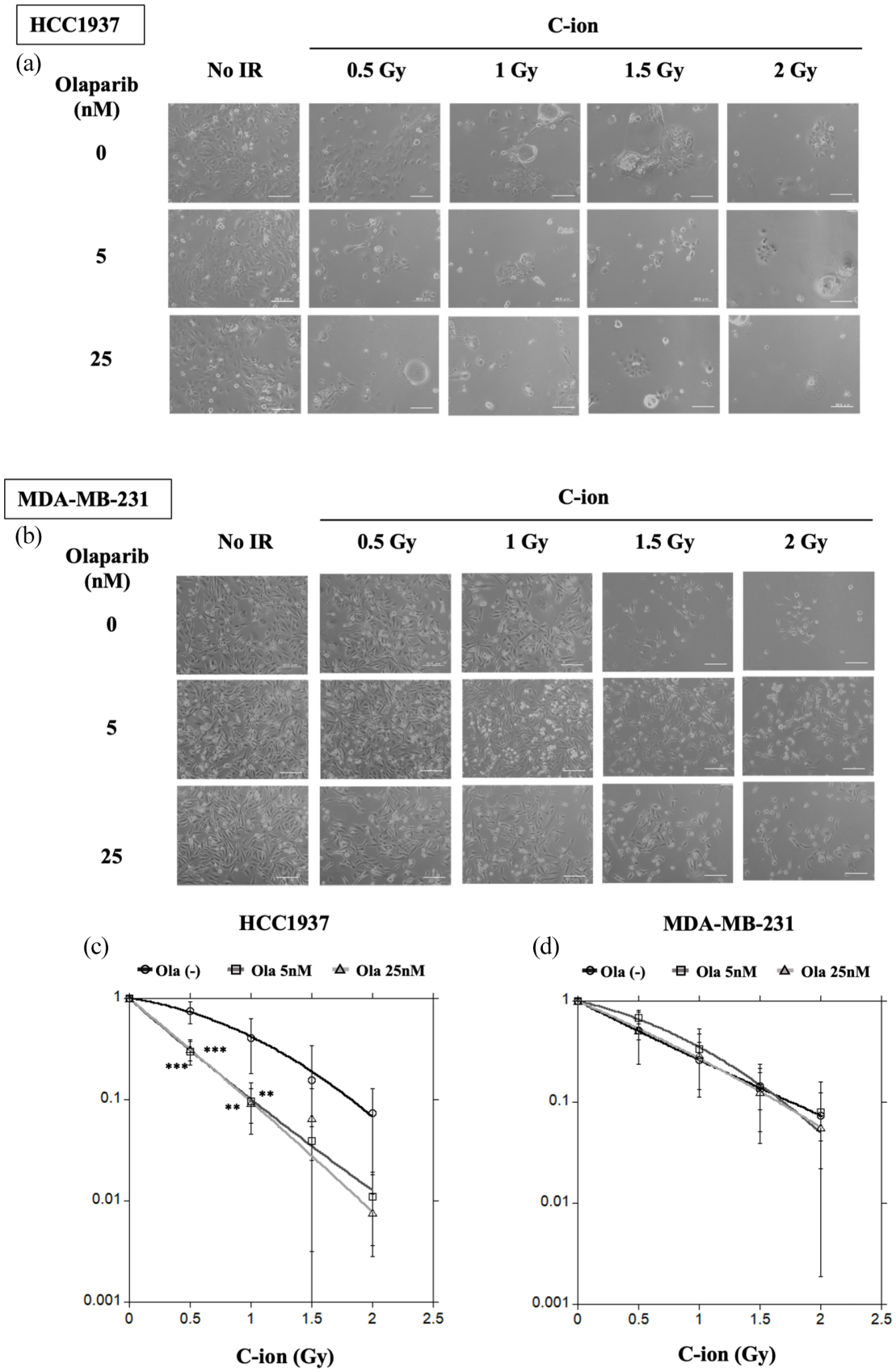
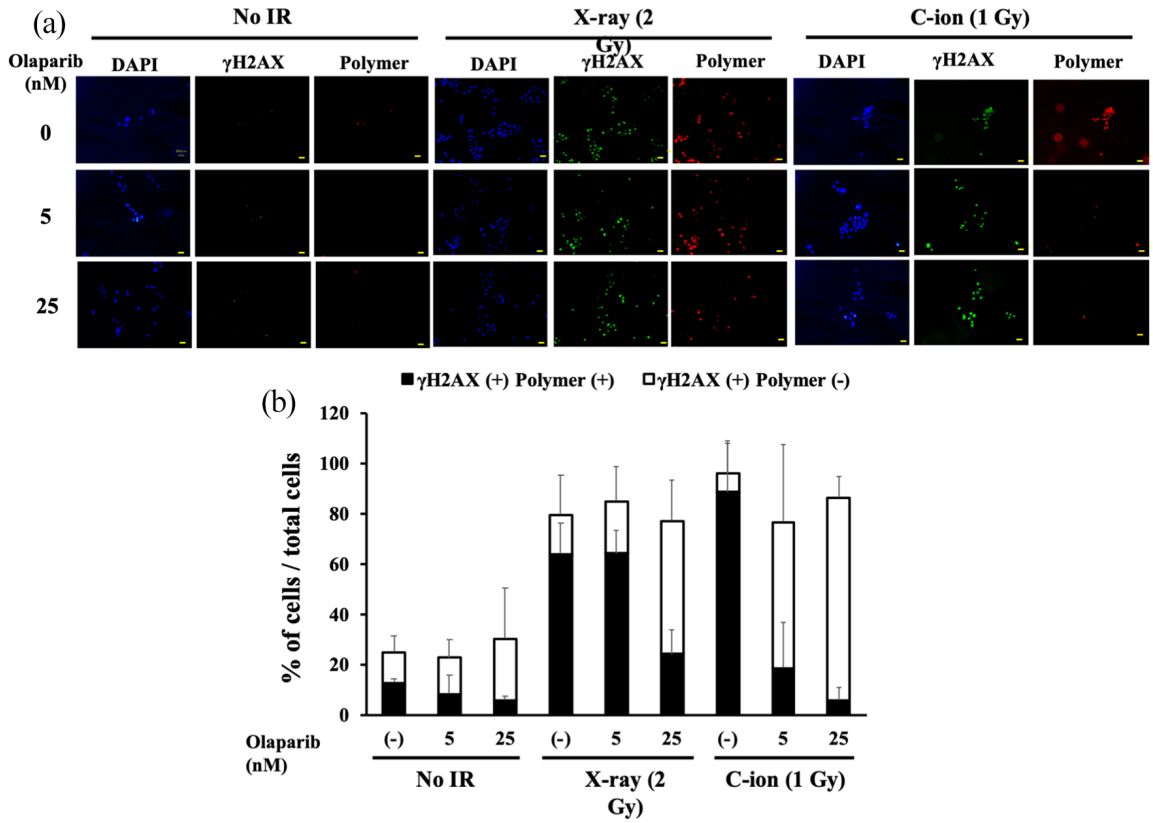


Figure 3. Low dose of olaparib showed effectiveness as a radio-sensitizer to C-ion radiation for HCC1937 but not for MDA-MB-231. Representative images of HCC1937 (A) and MDA-MB-231 (B) treated with C-ion beam irradiation and olaparib and corresponding HDS survival curves of HCC1937 (C) and MDA-MB-231 (D) were shown in graphs, respectively. Scale bar, 50 μm. Data in the graph show the mean ± SD of triplicate samples. $^{**}P < .01$, $^{***}P < .001$. For the statistical analysis, two-way ANOVA was performed using GraphPad Prism 8. ANOVA indicates analysis of variance; HDS, high-density survival; SD, standard deviation.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γH2AX (+) Polymer (+)
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% of cells / total cells
120
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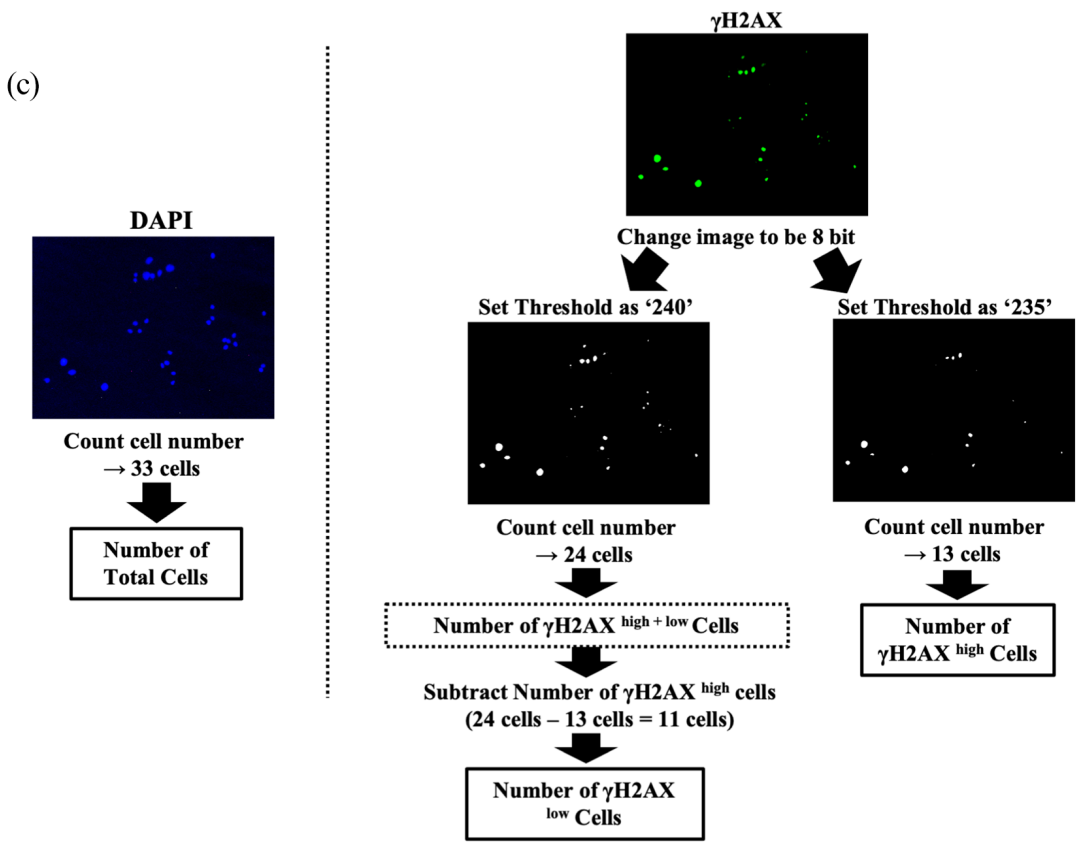
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→ 13 cells
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Number of γH2AX low Cells

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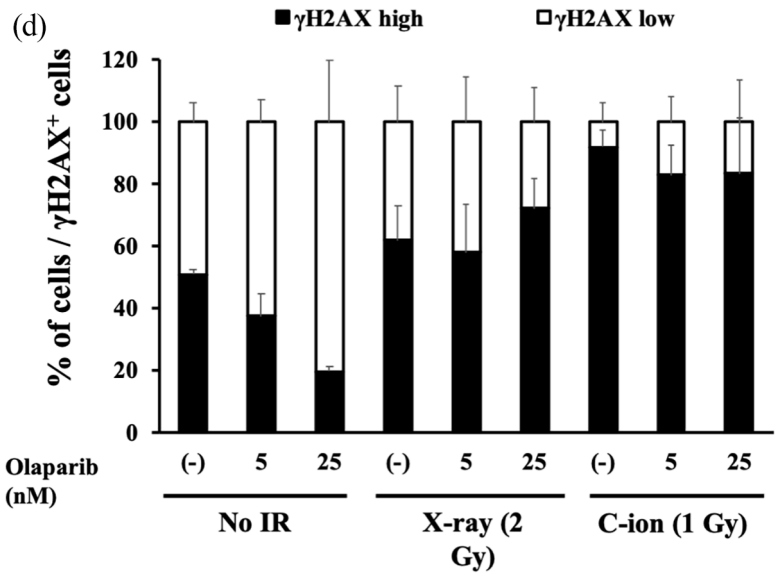

Figure 4. X-ray or C-ion radiation induced similar number of γH2AX-positive HCC1937 cells but induction levels were higher in C-ion irradiated HCC1937 with greater effectiveness of olaparib HCC1937 cells were irradiated with either 2 Gy X-rays or 1Gy C-ion beams followed by 6 h treatment with olaparib at 5 nM or 25 nM. Cells were then fixed and immunofluorescence labelled with anti-γH2AX antibody (the marker for DSB), and anti-PAR polymer antibody (the marker for PARP activity). Representative images of not irradiated, X-ray irradiated, or C-ion beams irradiated HCC1937 with olaparib treatment are shown in (A). Scale bar, 50 μm. (B) The number of γH2AX (+) with PAR polymer (+) cells (Black bar), or the number of γH2AX (+) with PAR polymer (–) cells (white bar) were counted and shown in graph. Data in the graph show the mean ± SD of triplicate samples. (C) Method used to count γH2AX^{high}, or γH2AX^{low} cells using the ImageJ software is shown. (D) γH2AX^{high} cells or γH2AX^{low} cells were counted and percent of each populations were shown in graph (Black bar shows γH2AX^{high} cells, and white bar represents γH2AX^{low} cells, respectively). Data in the graph show the mean ± SD of triplicate samples. For the statistical analysis, unpaired Student's *t*-test was performed using GraphPad Prism 8. DSB indicates double strand breaks; PARP, Poly (ADP-ribose) polymerase; SD, standard deviation.

middle panel in Figure 4A and B: C-ion with Ola (5 nM) vs X ray with Ola (5 nM), $P = .001$). Overall, the results indicate that PARP plays an important role in the DSB repair system (NHEJ) and is highly activated especially in C-ion irradiated HCC1937, and thus, even a moderate dose of PARPi, olaparib (5 nM), could exert significant cell killing in C-ion irradiated HCC1937 as we have observed in Figure 3C.

We show that higher level of PARP activity was induced in 1 Gy C-ion than 2 Gy X-ray irradiated cells; however, at this point, the mechanism involved is still unclear because the number of γH2AX-positive cells were comparable between 1 Gy C-ion and 2 Gy X-ray irradiation (Figure 4B), suggesting that the number of DSB-positive cells were similar between these two groups. To delineate this discrepancy in PARP activity, we further evaluated the difference in expression levels of γH2AX in each group as opposed to simply counting the number of γH2AX-positive cells (Figure 4C). As a result, most γH2AX-positive cells found in 1 Gy C-ion irradiated HCC1937, which was 81% of the total number of γH2AX-positive cells, were γH2AX^{high}, whereas only 48% of γH2AX-positive cells were γH2AX^{high} in 2 Gy X-ray irradiated cells (Figure 4D: Black bar of C-ion with Ola(-) vs Black bar of X-ray with Ola(-), respectively). Thus, these data suggest that although the number of γH2AX-positive cells induced via 1 Gy C-ion or 2Gy X-ray radiation was similar, their induction of γH2AX, γH2AX^{high} or γH2AX^{low}, was apparently different between these radiation types;.1 Gy C-ion radiation efficiently induced higher number of γH2AX^{high} cells than 2 Gy X-ray radiation did. Higher induction of γH2AX in C-ion irradiated HCC1937 indicated the greater number of DSB within the cell, and subsequent induction of NHEJ machinery. Greater activation of NHEJ may lead to increased activation of PARP as we have observed in C-ion irradiated HCC1937, thus making PARP inhibition using olaparib, very effective in killing these cells.

Discussion

In this study, we used TNBC cell lines, MDA-MB-231 (BRCA wt) or HCC1937 (BRCA mut), to investigate the effectiveness of PARPi, olaparib, as a radio-sensitizer for X-ray or C-ion beam. Irradiation of these cells leads to severe DNA damage and hence, activation of the DNA repair system is critical for cell survival. However, cells such as HCC1937, harboring a BRCA mutation are unable to activate HR, hence, these cells use alternative repair pathways, such as NHEJ. Here, we show that 1 Gy C-ion radiation exerted significant induction of DSBs, leading to γH2AX^{high} cells, in HCC1937 (BRCA mut) with significant activation of PARP. Such noticeable activation of PARP was not observed in X-ray irradiated HCC1937. As a result, the effectiveness of PARPi, olaparib,

was observed specifically in C-ion irradiated HCC1937 compared to X-ray irradiated HCC1937.

Poly (ADP-ribose) polymerase 1 is an essential factor for SSB repair.[33] Trapping PARP at SSB via olaparib, can cause the impairment in replication forks leading to induction of one-ended DNA DSB.[28] It is known that such one-ended DNA DSB can only be repaired by HR, thus olaparib is cytotoxic to BRCA1/2 mut cancers, whose HR machinery is defective as a result of the BRCA1 gene mutation.[7,13] Irradiation of cells can induce both SSB and DSB.[34] Thus, olaparib treatment of these irradiated cells leads to accumulation of DSBs because olaparib inhibits the SSB repair mechanism concurrently leading to the formation of more DSBs from unrepaired SSBs. However, notably, results in this study showed that olaparib treatment of X-ray or C-ion irradiated HCC1937 did not induce additional DSB positive cells (γH2AX-positive cells) (Figure 4A and B); treatment of irradiated cells with 5 nM olaparib or 25 nM olaparib did not increase the total number of γH2AX-positive cells (DSB-positive cells) within total imaged cells (Middle and Lower panel of green staining in Figure 4A, and Ola(5 nM) or (25 nM) versus Ola(–) in Figure 4B). Concomitantly, additional induction of $\gamma H2AX^{high}$ cells upon olaparib treatment was also absent in these irradiated HCC1937 (Figure 4D). Thus, the activated PARP induced in these irradiated cells may have another function other than SSB repair. It has been recently reported that PARP1 also plays a significant role in DSB repair via NHEJ.[10] To proceed with DNA repair using the NHEJ machinery, alterations in chromatin structure are required to promote the assembly of repair complexes on broken DNA. This alteration of chromatin structure is known to be initiated by PARP associated with DNA damage, which then recruits the chromatin remodeler, CHD2, through a poly(ADP-ribose)-binding domain.[8,10] Interestingly, we have found in this study that X-ray or C-ion radiation can induce similar number of γH2AX-positive HCC1937 cells, but these induction levels were higher in C-ion irradiated HCC1937. Consistently, PARP activation was also higher in these C-ion irradiated HCC1937 compared to that in X-ray irradiated cells. Therefore, it was indicated that C-ion induced significant DSBs, causing HCC1937 (BRCA mut) cells to repair DSB via NHEJ with PARP function, thus PARP was highly activated specially in C-ion irradiated HCC1937. Thus, olaparib could show greater effectiveness as a radio-sensitizer especially for C-ion irradiated HCC1937. In the case of X-ray irradiated HCC1937, no significant differences in PARP activation were observed with 5 nM olaparib treatment; the proportion of Polymer (+) cells within total cells as well as the intensity of polymer staining within each cells were unchanged between X-ray Ola(–) group and X-ray Ola (5 nM) group (data not shown). Thus, further experiments were needed to clarify the reason why the effects of 5 nM olaparib were absent specially in 2 Gy X-ray irradiated cells.

Recently, Ma et al have reported that inhibiting NHEJ pathway could significantly enhance radiosensitivity of human cancer cells to C-ion irradiation, rather than blocking HR pathway,[35] suggesting that NHEJ may be the significant DNA repair pathway for C-ion irradiated. NHEJ functions via two sub-pathways, the fast D pathway and the relatively slow B pathway.[33] Analysis of DSB rejoining using gel electrophoresis revealed fast components as well as slower components of NHEJ; the fast components include DNA-PKcs, Ku70/Ku80, DNA ligase IV, or XRCC4, and form a part of classical NHEJ (D-NHEJ), whereas, if any of these factors delays processing, cells alternatively use the slowly operating, backup pathway (B-NHEJ) to repair DSBs.[33,36,37] It is known that D-NHEJ is the main arm of NHEJ activated in mammalian cells,[33] whereas B-NHEJ is likely to be an evolutionarily older pathway that rejoins DNA ends with kinetics of several hours.[38] Interestingly, however, it has been reported that irradiation of mammalian cells can induce both D-NHEJ and B-NHEJ pathways for DSBs repair.[33,38] Thus, it is possible that both NHEJ sub-pathways may be activated in irradiated HCC1937 cells. As mentioned earlier, PARP1 has a role in altering chromatin structure to initiate NHEJ, but importantly, additional significance of PARP1 in B-NHEJ has also been reported.[33] In B-NHEJ pathway, activated PARP1 is thought to bind to the site of DSB and recruit several DNA repair genes including DNA damage sensors meiotic recombination 11 (Mre11) and Nijmegen breakage syndrome protein 1 (Nbs1) to the sites of DSBs. Mre11 then interacts with Rad50 and form MRN (Mre11-Rad50-Nbs1) complex, which proceeds to DSB repair via B-NHEJ.[39,40] In this study, we have not investigated whether such B-NHEJ or D-NHEJ were functional in C-ion irradiated HCC1937. But, it is clear that C-ion significantly induced γH2AX, with noticeable PARP activation. Thus, it is possible that NHEJ with both B-NHEJ and D-NHEJ sub-pathways were highly activated in C-ion irradiated HCC1937 as reported in other irradiated cells,[38] which induced drastic PARP activation to regulate chromatin structure for initiating NHEJ, as well as proceeding with the B-NHEJ sub-pathway.[33] It would be intriguing to further study whether NHEJ and its sub-pathways were highly activated in BRCA mut TNBC upon irradiation, and it would also be interesting to further study the difference in NHEJ activation, with regard to B or D sub-pathway activation, between C-ion and X-ray irradiated cells.

Poly (ADP-ribose) polymerase 1 is overexpressed in a variety of cancers, including ovarian, prostate and BCs as well as glioblastomas,[41-47] and PARP inhibitors, such as olaparib, are effective for the treatment of ovarian and BCs with BRCA1/2 mut.[47,48] From this study, we find that C-ion irradiation can exert significant amount of DSB in such BRCA mut TNBC with high PARP activation. Thus, PARPi, olaparib, would be a promising candidate as a radio-sensitizer for BRCA mut TNBC treatment especially with C-ion RT.

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