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Fractionated radiation exposure enhances the DNA repair capacity to acquire radioresistance in HCT8 human colorectal cancer cells

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► ***Original article***

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ABSTRACT

***Background*:** Fractionated radiotherapy is widely used for cancer treatment because of its advantages in the preservation of normal tissues; however, it may amplify the radioresistance of cancer cells. In this study, we aimed to understand whether and how fractionated radiation exposure induces radioresistance. ***Materials and Methods*:** HCT8 human colorectal cancer cells received a total X-ray dose of 5 Gy in either a single treatment (5 Gy administered once) or via fractionated exposure (1 Gy/day treatment for 5 consecutive days). We then examined the radioresistance of cancer cells exposed an additional 2 Gy X-ray by clonogenic assay and Western blot analysis. ***Results*:** Cells receiving fractionated exposure showed significantly greater proliferation and clonogenicity than those that received a single dose. Compared with the levels in the intact cells without radiation exposure, the expression levels of γH2AX, phospho-ATM and PARP were significantly enhanced only in the cells exposed to fractionated radiation. In contrast, the expression of cyclin D1 and cyclin E1 was enhanced only in the cells that received a single dose. In addition, the expression of SOD1 and SOD2 was slightly increased in the cells that received either the fractionated exposure or single exposure treatment. ***Conclusions*:** Fractionated radiation exposure facilitates radioresistance in HCT8 human colorectal cancer cells predominantly by enhancing their DNA repair capacity.

INTRODUCTION

Radiotherapy is an effective palliative and curative tool for malignant tumors, and approximately 50% of cancer patients receive radiotherapy [1]. Usually, malignant tumor growth is controlled via radiotherapy when a sufficiently high dose of radiation can be delivered. Unfortunately, the radiation dose is largely limited by the tolerance of patients [2] because therapeutic radiation to a solid tumor also damages normal tissue cells surrounding the tumor.

Radioresistance leads to the relatively low susceptibility of exposed cells to damage from additional radiation exposure. Therefore, the radioresistance of cancer cells is a major problem in the use of radiotherapy. The radioresistance of cancer cells can be either ‘‘intrinsic’’ or ‘‘acquired’’. Intrinsic radioresistance is an inherent condition in some cancer cells. In contrast, acquired radioresistance is induced by radiation treatment, which causes cancer cells to adapt to the effects of the radiotherapy [3, 4]. Acquired radioresistance is a main factor leading to the failure of radiotherapy [5]. Complex mechanisms, including cell survival regulators, DNA repair systems, and hypoxia signaling pathways, are involved in the radioresistance of cancer cells [6-9]. However, the precise mechanism of radioresistance, especially acquired radioresistance following radiotherapy, is not yet completely understood.

Fractionated radiation exposure is the most common technique used to increase the total dose of radiotherapy while selectively sparing healthy tissue cells, but the repeated radiation exposure involved in this strategy may may induce radioresistance cancer cells [10]. Many clinical trials have been focused on comparing the outcomes of different radiotherapy regimens (hypofractionated *vs.* standard fractionated radiation exposure and hypofractionated or standard fractionated *vs.* hyperfractionated radiation exposure) in cancer patients [11-13], but the results have been controversial. Therefore, it is critical to know whether and how fractionated radiotherapy facilitates cancer cells radioresistance acquisition.

In this study, we treated HCT8 human colorectal cancer cells with a total of X-ray dose of 5 Gy delivered via fractionated exposure (1 Gy × 5 days) or single exposure (5 Gy × 1 day) and then compared the biological characteristics of the cells, especially their radiosensitivity. Previous studies have proven

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that radiation-resistant cell lines can be obtained by repeated radiation exposure for a long time [14-16], but the relevant mechanisms have been inadequately investigated. Our data showed that daily 1 Gy exposure for only 5 days clearly induced radioresistance in cancer cells, predominantly by enhancing cellular DNA repair capacity.

MATERIALS AND METHODS

Cell culture

HCT8 human colorectal cancer cells from the American Type Culture Collection (ATCC) were used for the experiments [17]. Cells were maintained in RPMI-1640 medium (Wako Pure Chemical Industries Ltd, Osaka, Japan) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco/Life Technologies, Grand Island, USA). The cells were cultured at 37 °C in a humidified atmosphere with 5% carbon dioxide and 95% air.

Irradiation

Irradiation was administered using an ISOVOLT Titan-320 X-ray unit (200 kV, 15 mA, 5 mm aluminum filtration, GE Sensing and Inspection Technologies, Billerica, USA). The cells were irradiated with a total X-ray dose of 5 Gy at 1.0084 Gy/min. Fractionated exposure involved 1 Gy of X-rays for 5 consecutive days, but a single exposure was delivered in a 5 Gy dose on the first day. Cells without radiation exposure were used as controls.

Clonogenic assay

A clonogenic assay was performed to evaluate the radiosensitivity of cells. We seeded cells into six-well plates at a density of 100 cells/well. After overnight incubation, the cells were irradiated as indicated above. Colonies that had formed 10 days after irradiation were quantified by counting the total number of colonies consisting of more than 50 cells.

MTT assay

Cell proliferation was evaluated using Cell Proliferation Kit I (#11465007001, Roche Life Science, USA). Briefly, cells were seeded in 96-well culture plates (5×10^3 cells/well). After 24 hours of culture, the cells were irradiated with a 2 Gy dose of X-rays. We added 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) to the cell culture medium 24 hours later, and then, incubated the cells for another 4 hours. The formation of formazan from MTT was stopped by adding a solubilization agent. The absorbance was measured at 570 nm using a microplate reader (Multiskan Fc, Thermo Fisher Scientific, USA).

Western blotting

Following radiation exposure as indicated, the cells were washed with phosphate-buffered saline (PBS) and lysed at 4 °C in lysis buffer. The insoluble material was removed by centrifugation at 15,000 × g for 15 minutes. Total protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk for 1 hour and incubated overnight with primary antibodies against H2A histone family member X phosphorylated at serine 139 (γH2AX) (1:500 dilution, #ab2893, Abcam, UK), Ki67 (1:1000 dilution, #ab16667, Abcam, UK), cyclin D1 (1:1000 dilution, #2978s, Cell Signaling Technology, USA), cyclin E1 (1:1000 dilution, #4129, Cell Signaling Technology, America), phospho-ataxia telangiectasia mutated kinase pS1981 (pATM) (1:1000 dilution, #2152-1, Epitomics, UK), poly (ADP-ribose) polymerase (PARP) (1:1000 dilution, #9542, Cell Signaling Technology, USA), superoxide dismutase 1 (SOD1) (1:1000 dilution, #sc-11407, Santa Cruz Biotechnology, USA), superoxide dismutase 2 (SOD2) (1:1000 dilution, #sc-30080, Santa Cruz Biotechnology, USA), and α-tubulin (1:1000 dilution, #3873S, Cell Signaling Technology, USA). After washing, the membranes were incubated for 1 hour with the appropriate HRP-conjugated secondary antibody (1:1000 dilution, DAKO, USA) at room temperature. The membranes were washed, and blots were visualized using an enhanced chemiluminescence kit (#RPN2106, GE Healthcare Life Sciences, USA). Semiquantitative analysis was performed by measuring the density of the bands with an ImageQuant LAS 4000 Mini biomolecular imager (GE Healthcare Life Sciences, USA).

Statistical analysis

All data are presented as the mean ± SD. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's test (GraphPad Prism 9.3.0, USA). A p value less than 0.05 was considered to be statistically significant.

RESULTS

Fractionated radiation exposure preserves the viability and clonogenic ability of cancer cells

To test radiosensitivity, we irradiated cells with X-rays and then evaluated cell viability and clonogenicity. As shown in the representative images (figure 1A), radiation exposure resulted in a reduction in cell number, which was lower than that of the control cells (0 Gy) (figure 1B). Interestingly, compared to the single-exposure dose (5 Gy × 1), the fractionated exposure (1 Gy × 5) treatment resulted in significantly increased cell survival ($P < 0.05$, figure 1A, B). We irradiated the cells daily with 2 Gy of X-rays for 5 days (a total dose of 10 Gy) and observed even higher cell density and cell number than that of the cells treated with the single-exposure exposure

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regimen, a total dose of 5 Gy (figure 1A, B). An MTT assay also revealed that the proliferative activity of the cells after fractionated exposure was significantly higher than that of the cells that received the single-exposure treatment (P < 0.05, figure 1C). Similarly, the clonogenicity assay showed that a single exposure to 5 Gy resulted in a significantly fewer colonies than were counted after fractionated exposure (P < 0.05, figure 1D, E). These findings suggest that fractionated radiation exposure preserves the viability and clonogenicity of cancer cells.

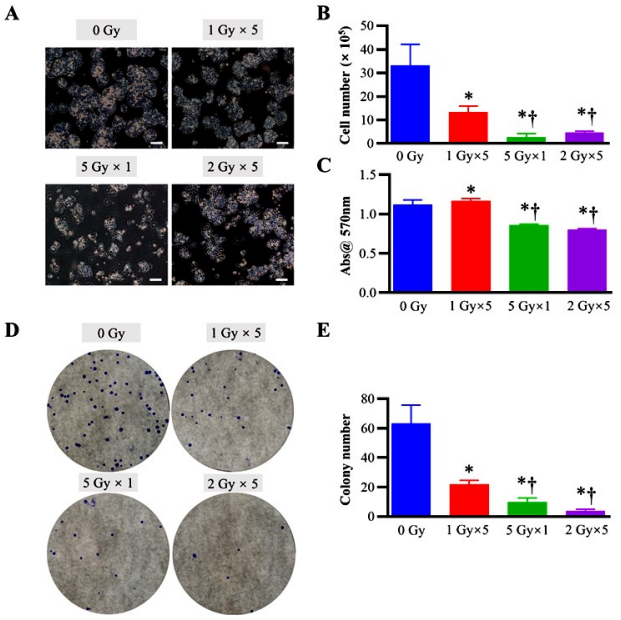

Figure 1. Cell survival and clonogenicity of HCT8 cells after different treatments. **A)** Representative images of cell proliferation obtained with a phase contrast microscope (scale bars= 200 μm) and **B)** the total cell number on Day 5 are shown. **C)** MTT assay of the proliferative activity of cells on Day 5. **D)** Representative images showing the clonogenic assay results and **E)** the quantitative data on colony formation 10 days after irradiation. The data represent the mean ± SD from three independent experiments. *P < 0.05 vs. 0 Gy, †P < 0.05 vs. 1 Gy × 5.

Fractionated radiation exposure facilitates radioresistance acquisition by cancer cells

To investigate the acquisition of radioresistance, cells irradiated in a fractionated exposure or single exposure treatment to receive a total dose of 5 Gy were harvested on Day 5 and then exposed to an additional 2 Gy dose of radiation (figure 2A). As expected, the clonogenic assay showed that significantly more colonies formed from cells irradiated via fractionated exposure than from cells irradiated in a single exposure (figure 2B, C). An MTT assay also showed significantly higher proliferative activity of cells irradiated via fractionated exposure than those receiving a single exposure (figure 2D). These data suggest that fractionated radiation exposure facilitates the acquisition of radioresistance by cancer cells.

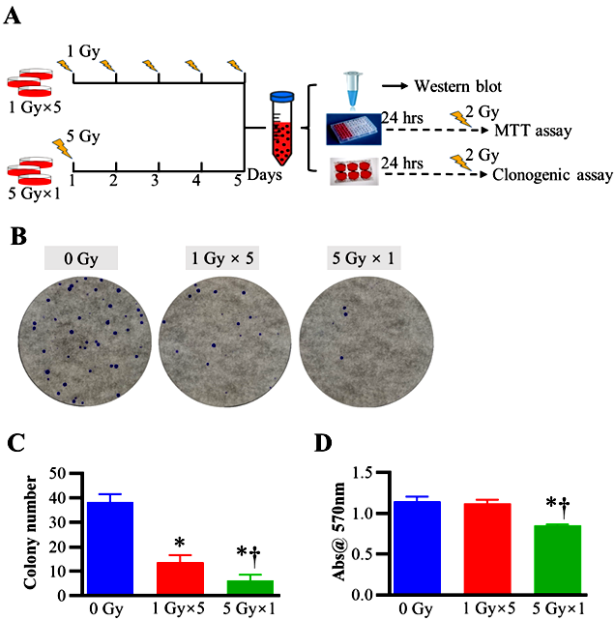

Figure 2. Radiosensitivity of cancer cells that received 5 Gy of X-rays via fractionated exposure or in a single dose. **A)** Scheme showing the radiation doses and assessments. **B)** Representative images of the clonogenicity assay results and **C)** quantitative data on colony formation. **D)** MTT assay of the proliferative activity of irradiated HCT8 cells after exposure to an additional 2 Gy dose. The data represent the mean ± SD from three independent experiments. *P < 0.05 vs. 0 Gy, †P < 0.05 vs. 1 Gy x 5.

Fractionated radiation exposure enhances the DNA repair capacity of cancer cells

To further understand the underlying mechanism, we evaluated the expression of DNA repair molecules, proliferation markers, cell cycle regulators, and antioxidant enzymes in cells that received a 5 Gy dose of X-rays via fractionated exposure or in a single dose (figure 2A).

Compared to that in the intact cells without radiation exposure, the expression of γ-H2AX, pATM and PARP was significantly enhanced in cells that received 5 Gy of X-rays via fractionated exposure (P < 0.05 *vs.* 0 Gy, Figure 3) but did not significantly change in cells that received a total 5 Gy dose of X-rays in a single exposure. However, the expression of cleaved PARP was significantly increased in the cells that received 5 Gy X-ray via either fractionated exposure or in a single dose (p < 0.05 *vs.* 0 Gy, figure 3).

The expression of Ki67, an important proliferation marker, was significantly reduced in cells that received 5 Gy X-ray in a single dose (P < 0.05 *vs.* 0 Gy, Figure 4) but not by fractionated exposure. In contrast, the expression of cyclin D1 and cyclin E1, two of the most important cell cycle regulators, was significantly enhanced in cells that received 5 Gy X-ray in a single dose (P < 0.05 *vs.* 0 Gy, Figure 4) but not by fractionated exposure.

Compared to that in the intact cells without radiation exposure, the expression of SOD1 and

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SOD2, two important antioxidant enzymes, was only slightly increased in the cells that received 5 Gy X-ray via either fractionated exposure or in a single dose (figure 5).

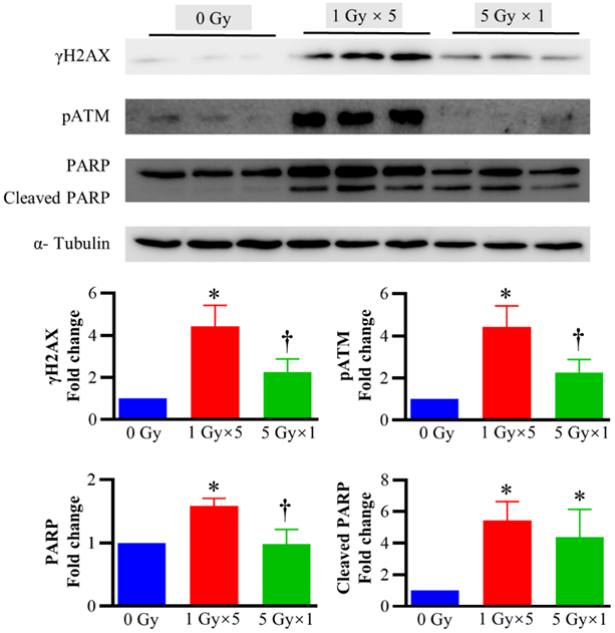

Figure 3. Western blot analysis of the expression of γH2AX, pATM, and PARP (cleaved PARP). Representative blot (upper images) and semiquantitative data (lower bar graphs) show the expression of γH2AX, pATM, and PARP (cleaved PARP) in cells that received 5 Gy X-ray via fractionated exposure (1 Gy × 5) or in a single dose (5 Gy × 1). Cells without radiation exposure (0 Gy) were used as controls. The data represent the mean ± SD from three independent experiments. *P < 0.05 vs. 0 Gy, †P < 0.05 vs. 1 Gy × 5.

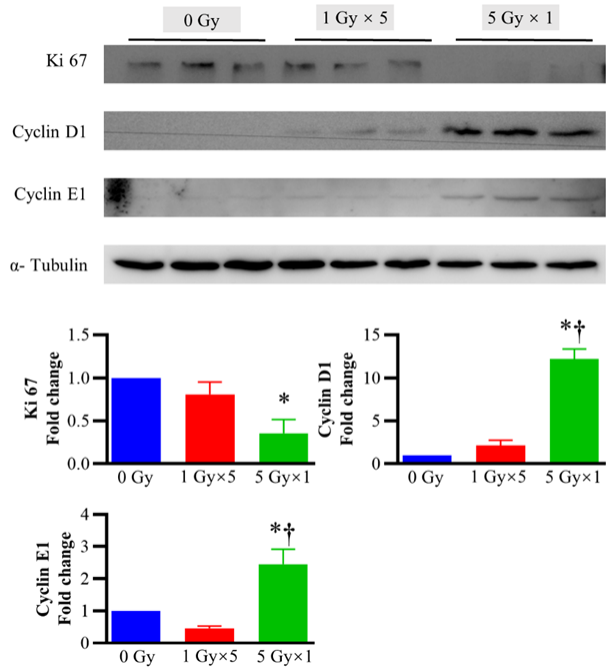

Figure 4. Western blot analysis of the expression of Ki67, cyclin D1, and cyclin E1. Representative blot (upper images) and semiquantitative data (lower bar graphs) show the expression of Ki67, cyclin D1, and cyclin E1 in cells that received 5 Gy of X-rays by fractionated exposure (1 Gy × 5) or in a single dose (5 Gy × 1). Cells without radiation exposure (0 Gy) were used as controls. The data represent the mean ± SD from three independent experiments. *P < 0.05 vs. 0 Gy, †P < 0.05 vs. 1 Gy × 5.

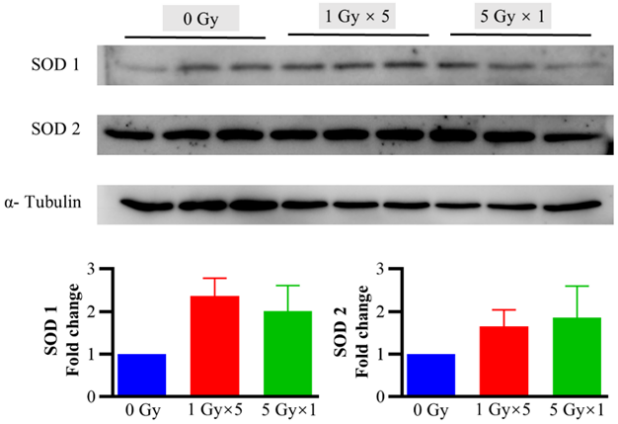

Figure 5. Western blot analysis of the expression of SOD1 and SOD2. Representative blot (upper images) and semiquantitative data (lower bar graphs) show the expression of SOD1 and SOD2 in cells that received 5 Gy of X-rays by fractionated exposure (1 Gy × 5) or in a single dose (5 Gy × 1). Cells without radiation exposure (0 Gy) were used as controls. The data represent the mean ± SD from three independent experiments.

DISCUSSION

Fractionated radiotherapy is widely used for cancer patient treatment, but cancer cells may acquire radioresistance with repeated radiation exposures [14-16]. In the present study, we investigated whether and how fractionated radiation exposure amplifies radioresistance in cancer cells. Wild-type p53-expressing HCT8 human colorectal cancer cells were used in this study. HCT8 cells are frequently used for experiments, including radiation-related studies [19, 20]. HCT8 cells exhibit relatively poor response to radiation exposure, and radioresistance can be easily and quickly induced in HCT8 cells [21]. According to our data obtained from MTT and clonogenic assays, fractionated radiation exposure clearly amplified radioresistance in HCT8 cells.

Complex mechanisms, such as DNA repair [22], cell cycle arrest [23], and redox regulation [24], are involved in the radioresistance of cancer cells. In addition to cancer cells, the tumor microenvironment, such as cancer-associated fibroblasts and immune cells, are associated with radiosensitivity [28]. Previous studies have demonstrated that cell senescence, the epithelial -mesenchymal transition and the activation of the nuclear factor-kappa B pathway contribute to the radioresistance of cancer cells following fractionated radiation exposure [25, 26]. According to a previous *in vitro* investigation, cancer cells acquired radioresistance within 2-3 weeks of fractionated radiotherapy, but the acquired radioresistance will be recover in 6 weeks after the discontinuation radiotherapy [26]. In contrast to previous studies, we compared the radioresistance acquisition of cancer cells after an additional 2 Gy treatment following exposure to a single dose (5 Gy × 1 day) or to fractionated exposure (1 Gy × 5 days). We examined the expression of DNA repair molecules, cell cycle

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regulators, and antioxidant enzymes in cancer cells. Radioresistance can be caused by all available DNA repair pathways [7]. Several DNA repair proteins, including PARP, ATM, and γH2AX, are quickly recruited to damaged DNA sites [27]. Enhanced DNA repair capacity can effectively increase cell survival [27]. Several compounds targeting DNA repair are being developed to attenuate the acquisition of radioresistance of cancer cells following fractionated radiotherapy [28]. In our study, the expression of PARP, γ-H2AX and pATM was clearly enhanced in HCT8 cells that were irradiated by 5 Gy of X-rays via fractionated exposure, but not after exposure to a single dose of 5 Gy. These findings suggested that fractionated radiation exposure likely amplifies radioresistance by increasing DNA repair capacity, although the role of ATM activation in initiating increased radioresistance remains controversial [29].

Radiosensitivity varies widely depending on the cell cycle phase [30]. Ki67 is highly expressed in cycling cells, but its expression is profoundly downregulated in resting G0-phase cells [31]. Cell proliferation is always inhibited after cell cycle arrest. After the cell is exposed to ionizing radiation, the typical cell cycle is interrupted to allow sufficient time for DNA repair or to prepare for the cell death or senescence program in the case of extreme or irreparable damage [32]. As cell cycle regulators, cyclin D1 and cyclin E1 play important roles in cancer progression, but their roles in radioresistance acquisition remain unclear. Cyclin D1 overexpression has been reported to disrupt DNA replication and induce replication-associated DNA double-strand breaks [33, 34]. Targeting the cyclin D1/cyclin-dependent kinase 4 signaling pathway effectively eradicated the radioresistance of malignant tumors followed by fractionated radiotherapy [18, 34]. Although cell cycle redistribution is a classical phenomenon of cancer cells after radiation exposure, cell cycle distribution seems to change marginally in the first few days following fractionated radiation treatment [35]. As we purified the total protein from the irradiated cells for Western blot analysis at different time points after the last exposure between groups (Figure 1D), it was very difficult to interpret the expression of cyclin D1 and cyclin E1 in radioresistance acquisition in this study.

Reactive oxygen species constitute a group of short-lived highly reactive and oxygen-containing molecules that can induce DNA damage and affect the DNA damage response. As ionizing irradiation can produce reactive oxygen species that damage cells, previous studies have demonstrated a relationship between radioresistance and the expression of antioxidant enzymes in cancer cells [36, 37]. In contrast to previous studies, the expression of SOD1 and SOD2 was not significantly induced in HCT8 cells by either fractionated exposure or single exposure to 5 Gy of X-rays. According to our data, redox regulation is not extensively involved in the acquisition of radioresistance by HCT8 cells following fractionated exposure.

The reason for the different findings among studies remains unclear. The acquisition of radioresistance is a very complicated process. There is still an absence of consensus on the mechanism involved in radioresistance, especially the amplification of radioresistance following fractionated exposure. In fact, the dose and the interval between the administrations of each dose in the fractionated exposure regimen is critical to the outcome of radiotherapy [38, 39].

In summary, our experimental data suggest that fractionated radiation exposure facilitates acquired radioresistance by cancer cells, predominantly through the enhancement of DNA repair capacity, not the alteration of the cell cycle or redox regulatory mechanisms. However, *in vivo* studies and clinical trials are clearly needed to confirm our findings.

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Consent for publication: Not applicable.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

Authors' contributions: T-S L is the creator, directing experimental design, K H performed the major of experiments and wrote the manuscript. M O partly performed clonogenic assay and MTT assay. C Y participated in data analysis and manuscript revision. L A participated in experimental design and data curation. X Z participated in data curation. All authors read and approved the final manuscript.

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