

Original Article

Function and mechanism of combined PARP-1 and BRCA genes in regulating the radiosensitivity of breast cancer cells

Wei Zhao^{1,2*}, Hongbo Hu^{1,3*}, Qiyao Mo¹, Ying Guan¹, Ye Li¹, Youqing Du¹, Ling Li¹

¹Department of Radiation Oncology, Affiliated Tumor Hospital of Guangxi Medical University, Nanning, Guangxi Autonomous Region, China; ²Guangxi Colleges and Universities Key Laboratory of Biological Molecular Medicine Research, Nanning, Guangxi Autonomous Region, China; ³Department of Oncology, The People's Hospital of Chongzuo City, Longxiashan Road, Chongzuo, Guangxi Autonomous Region, China. *Co-first authors.

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Abstract: Objective: To study the function and mechanism of combined PARP-1 and BRCA genes in regulating the radiosensitivity of breast cancer cells by poly ADP-ribose polymerase-1 (PARP-1) inhibitor 3-aminobenzamide (3-AB) on BRCA mutant and non-mutant breast cancer cells. Methods: Four groups of BRCA mutant cells MDA-MB-436 and BRCA non-mutant cells MDA-MB-231 were divided respectively into control (CTRL), ionizing radiation alone (IR), 3-AB alone (3-AB), and ionizing radiation combined with 3-AB (IR+3-AB) groups. The γ -H2AX foci were detected by immunofluorescence assay to show the DNA double-strand damage. The clonogenic cell survival assay was applied to evaluate the radiosensitivity of breast cancer cells, and flow cytometry was used to assess the percentage of apoptosis cells. Results: The apoptosis rate of MDA-MB-436 cells was significantly increased compared with MDA-MB-231 cells treated with irradiation, and 3-AB could further enhance the effect. Similarly, the result of γ -H2AX foci detection showed that DNA double-stranded damage of the MDA-MB-436 cells was significantly greater than that of MDA-MB-231 cells ($t = 4.57, P < 0.05$), and the DNA damage of MDA-MB-436 cells in IR+3-AB group was the most remarkable. The difference was significant ($t = 3.26, P < 0.05$). In the same group, compared with MDA-MB-231 cells, MDA-MB-436 cells had the significantly greater apoptosis rate ($t = 2.96, P < 0.05$). The apoptosis rate of MDA-MB-436 cells in the IR+3-AB group showed by flow cytometry was highest ($t = 3.81, P < 0.05$). Conclusions: Compared with non-BRCA mutant MDA-MB-231 cells, the BRCA mutant breast cancer MDA-MB-436 cells could incur significantly greater DNA damage, and therefore the radiosensitivity of MDA-MB-436 cells is higher than that of MDA-MB-231 cells. The inhibitor of PARP-1, which can block the repair of single-strand damage caused by radiation, can further enhance the level of apoptosis and radiosensitivity of BRCA-mutant cells.

Keywords: PARP-1 inhibitor, BRCA gene, radiosensitivity, breast cancer

Introduction

Breast cancer is a common malignant tumor in women, whose annual incidence in the world is almost 2%, and the incidence and mortality in China are also rising. Radiotherapy is an important component of treatment for breast cancer, and is also the main means of palliative treatment for advanced breast cancer. Studies have shown that radiotherapy works as adjuvant treatment after surgery and could reduce the local recurrence rate and death risk of breast cancer [1]. However, in the course of radiotherapy, cancer cells will produce resistance to

radiotherapy through a variety of mechanisms, and thereby affect the efficacy of radiotherapy. The capacity of DNA repair directly affects the radiosensitivity of tumor cells. PARP-1 and BRCA respectively control the repair of single strand and double strand DNA damage caused by irradiation and directly affect the sensitivity of tumor cells [2]. BRCA is closely related to the occurrence and development of breast cancer. Carriers of the BRCA mutation had a 80% prevalence of breast cancer in their lifetime, and 32% and 13% of breast cancers are found to be associated with mutations of BRCA1 and BRCA2, respectively. In this project, the phe-

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nomenon of synthetic lethality was used to study the role of PARP-1 inhibitor 3-AB in the radiosensitivity of breast cancer cells with BRCA gene mutation to explore the mechanism of action of PARP-1 and BRCA genes in DNA damage repair induced by irradiation in order to effectively enhance the radiosensitivity of BRCA mutant breast cancer.

Materials and methods

Reagents and cell culture

The BRCA mutant cells MDA-MB-436 and BRCA non-mutant cells MDA-MB-231 were purchased from the Typical Cell Culture Preservation Committee of Chinese Academy of Sciences (Shanghai, China). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and a combination of penicillin (50 U/ml) and streptomycin (50 µg/ml), and maintained at 37°C in a 5% CO₂ atmosphere humidified to 95-100%. High-energy linear accelerator (Precise 1120, Elekta Instrument AB, Stockholm, Sweden) was applied to provide 6 MV X-ray exposure. The source-tumor distance was SSD = 100 cm, and the dose rate was 200 cGy/min.

The PARP-1 inhibitor 3-amino benzamide (3-AB) was purchased from Sigma (St. Louis, USA), Annexin V-FITC was purchased from Bibo Company (Nanjing, China). The γ-H2AX monoclonal antibody and the secondary antibodies (goat anti-mouse IgG) were purchased from Cell Signaling Technology (Danvers, MA).

Flow cytometry assay

The cells were divided into four groups: Control group (CTRL group), 3-AB group (3-AB, 10 mmol/L), Irradiation group (IR group, 8 Gy) and irradiation combined 3-AB group (IR+3-AB group). Cells were grown in 6 well plates and treated with drug or irradiation. 48 hours after treatment, the cells were trypsinized to give a single-cell suspension, and were pelleted by centrifugation. Then the cells were washed twice in cold PBS buffer, fixed in ice-cold 70% ethanol for at least 24 h at -20°C. The ethanol was removed by centrifugation, and the cells were resuspended in 0.5 ml propidium iodide staining solution (0.1% sodium citrate, 0.3% Triton X-100, 100 mg/ml RNase A, 100 mg/ml propidium iodide) and incubated at 37°C for 30 min at a concentration of 5×10^5 cells/mL.

Cellular fluorescence was measured by FASort flow cytometry (Becton Dickinson, USA). The data were analyzed by CELL Quest software. Experiments were repeated three times.

Clonogenic assay

The cells were trypsinized and counted using a hemacytometer. In a typical experiment, 50 to 5,000 cells were plated per well of a six-well plate for irradiation with doses ranging from 0, 2, 4, 6, 8, and 10 Gy, respectively. The plates were then incubated at 37°C for 10 to 14 days; the cells were stained with Giemsa and colonies with more than 50 cells per colony were scored under a light microscope. Survival fraction under different doses was calculated based on the colonies according to dose. Based on a simple multi-target model, survival curves were constructed by plotting colony-forming ability on a logarithmic scale as a function of the radiation dose administered on a linear scale. Then the radiation biology parameter D0 value (mean lethal dose), Dq value (quasi-threshold dose), N value (target number) and SF2 (cell survival fraction on 2 Gy) were calculated. All experiments were repeated three times.

siRNA

The scrambled RNAi oligonucleotides and siRNAs targeting mouse Atg5, Atg7 (ONTARGETplus SMARTpool™ RNAi reagents) were obtained from Dharmacon (Layfayette, CO). All siRNAs were transfected into cells using the DharmaFECT 4 Transfection Reagent (Dharmacon, Layfayette, CO) according to the manufacturer's protocol siRNA effectiveness was validated by western blotting.

Western blot

At the end of the designated treatments, cells were lysed in M2 lysis buffer (20 mM Tris at pH 7, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM glycerol phosphate, mM sodium vanadate and proteinase inhibitor cocktail). An equal amount of protein was resolved by SDS-PAGE and transferred onto PVDF membrane (Bio-Rad). After blocking with 5% non-fat milk, the membrane was probed with designated first and second antibodies, developed with the enhanced chemiluminescence method (Pierce) and visualized

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Table 1. Apoptosis of MDA-MB-436 and MDA-MB-231 cells treated with radiation and 3-AB (%; $\bar{x} \pm S$)

Cell lines	N of samples	CTRL	3-AB	IR	IR+3-AB
MDA-MB-436	3	4.4 ± 0.52	10.2 ± 0.84	26.3 ± 2.37	38.5 ± 2.93
MDA-MB-231	3	4.1 ± 0.66	9.1 ± 0.95	18.8 ± 2.71	27.4 ± 2.52
t		0.17	0.83	2.96	3.81
P		> 0.05	> 0.05	< 0.05	< 0.05

CTRL, control group; 3-AB, 3-AB treatment group; IR, irradiation group; IR+3-AB, irradiation+3-AB group.

with the Kodak Image Station 440CF (Kodak). The band density was quantified using the ImageJ image processing program developed by NIH and normalized to that of the control group.

γ-H2AX detection

After 1 hour of treatment, cells were fixed in ice cold 50% CH₃OH and 50% (CH₃)₂CO for 20 minutes at room temperature. After fixation cells were permeabilized with 0.5% Triton X100: PBS and then blocked with 0.2% skimmed milk, 0.1% Triton-X 100, 5% FBS in Phosphate Saline Buffer (PBS). Cells were then stained with anti-γ-H2AX antibody (Upstate) and anti-mouse AlexaFluor-488 secondary antibody (Molecular Probes) for the kinetics experiments. Coverslips were mounted with VECTASHIELD® Mounting Medium containing DAPI, to counterstain cellular nuclei. γ-H2AX foci were scored manually by the same operator throughout the cell nuclei using a Zeiss Apotome fluorescence microscope with 63X objective and the average number of foci per cell was calculated from a minimum of 250 cells per dose/time point. Experimental data represent the average of 3 independent experiments.

Statistical analysis

All experiments were done in triplicate, and all data are presented as mean ± standard deviation (SD). Significance was determined by Student t-test.

Result

The effects of PARP-1 inhibitor 3-AB on the apoptosis of MDA-MB-436 and MDA-MB-231 cells

The result showed that the 3-AB alone had no significant effect on the apoptosis of these two

kinds of cells. Compared with the control group, the two groups treated with irradiation incurred more apoptosis, and at the same dose of irradiation, the apoptosis rate of MDA-MB-436 cells was significantly higher than that of MDA-MB-231 cells. The difference was statistically significant (t = 2.96, P < 0.05). For the

same cells, the apoptosis rate of the IR+3-AB group was significantly increased compared with the IR group. The difference was significant (t = 3.81, P < 0.05); especially obvious was the increased apoptosis rate of MDA-MB-436 cells (**Table 1**).

Effects of 3-AB on the radiosensitivity of MDA-MB-436 and MDA-MB-231 cells

After treatment, survival curves were obtained and radiosensitivity parameters were calculated using the multi-target single-hit model. The experimental results showed that the values of D₀, Dq, N, SER, and SF2 of MDA-MB-436 cells were less than that of MDA-MB-231 cells. The shoulder region of the cell survival curve of MDA-MB-436 cells was more narrow, suggesting that the radiosensitivity of BRCA mutant breast cancer is higher than that of BRCA non-mutant cells. After treatment, cells were irradiated with different doses. The results show that compared with the irradiation group, the shoulder region of survival curve, and D₀, Dq, N values of IR+3-AB group all decreased, suggesting that PARP-1 inhibitor 3-AB can increase the radiosensitivity of BRCA mutated (MDA-MB-231) and BRCA non-mutated breast cancer cells (MDA-MB-436), with marked increase of radiation sensitivity in MDA-MB-231 (**Figure 1**).

Difference of DNA double strand breaks (DNA-DSBs) between MDA-MB-436 and MDA-MB-231 cells

Two groups of cells were given or not given 2 Gy irradiation, and 1 h after irradiation, the γH2AX foci formation was detected by immunofluorescence. The results showed that in the two groups, the number of γH2AX foci was not significantly affected by the PARP inhibitor 3-AB alone. Compared with the non-irradiation group, irradiation can cause cell DNA double-

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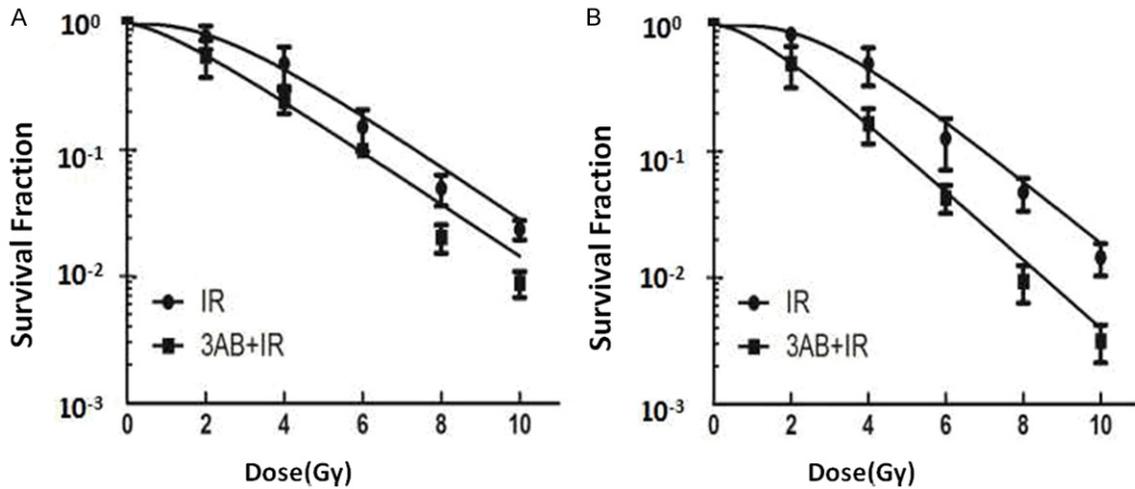


Figure 1. Cell Survival Curve of MDA-MB-231 cells (A) and MDA-MB-436 cells (B).

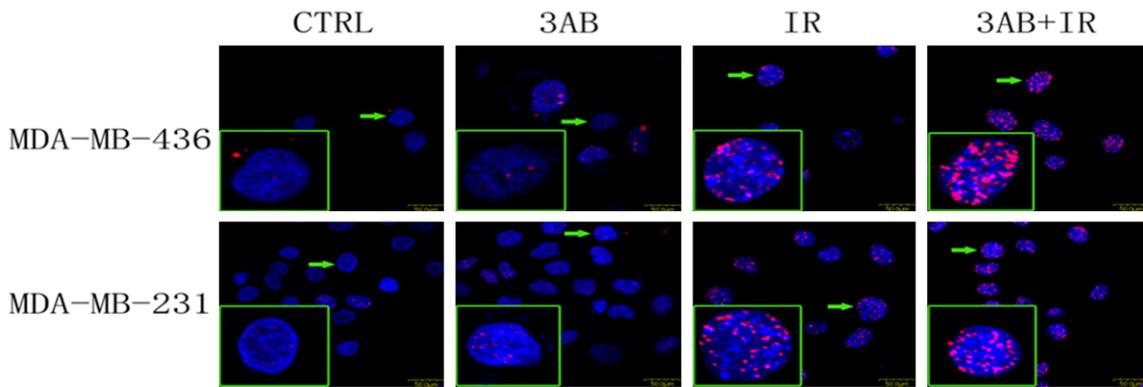


Figure 2. The γ H2AX foci of MDA-MB-436 and MDA-MB-231 cells were detected 1 hour after irradiation with 2 Gy X rays.

Table 2. The DNA-DSB of MDA-MB-436 and MDA-MB-231 cells treated with radiation and 3-AB (% , $\bar{x} \pm S$)

Cell lines	N of samples	CTRL	3-AB	IR	IR+3-AB
MDA-MB-436	100	1.3 \pm 0.25	2.5 \pm 0.54	38.3 \pm 4.51	46.5 \pm 5.74
MDA-MB-231	100	1.1 \pm 0.28	1.7 \pm 0.29	29.5 \pm 4.75	39.4 \pm 3.59
t value		0.29	0.65	4.57	3.26
P value		> 0.05	> 0.05	< 0.05	< 0.05

CTRL, control group; 3-AB, 3-AB treatment group; IR, irradiation group; IR+3-AB, irradiation+3-AB group.

strand breaks, and at the same dose, the γ H2AX foci of MDA-MB-436 cells was higher than that of MDA-MB-231 cells ($t = 4.57$, $P < 0.05$). For the same cells, the γ H2AX foci of the cells in IR+3-AB group is much higher than that of irradiation alone group ($t = 3.26$, $P < 0.05$); especially in MDA-MB-436 cells, the DNA-DSBs increased significantly (**Figure 2; Table 2**).

Discussion

Radiotherapy is an indispensable part in the comprehensive treatment of breast cancer. The difference in radiosensitivity determines therapeutic effect. If frequent DNA double-strand breaks caused by irradiation cannot be repaired, cells would undergo apoptosis.

PARP-1 and BRCA control the repair of single and double DNA strand pathways, which play a key role in the repair of DNA damage induced by irradiation, directly affecting the sensitivity of tumor cells [5, 6].

Because of the important role of PARP-1 in DNA repair, use of PARP-1 inhibitors occurs two

ways. The first is the PARP-1 for DNA inhibitors as a radiotherapy or chemotherapy sensitizing agent [7, 8]; the second is the specific genetic characteristics of some tumors by chemical synthetic lethality to cause DNA damage, but the overall treatment effect is not ideal [9]. Therefore, this study introduced the synthetic lethal phenomenon: namely, when inhibited PARP-1 coexists with a BRCA defect, a large number of DNA strand breaks induced by radiation will lead to synthetic lethality. The specific mechanism is: when PARP-1 protein of breast cancer cells is inhibited, frequent DNA-SSBs in the cells fail to be repaired in a timely way and would accumulate increasingly, which causes the disintegration of the replication fork, and finally produces large amounts of DNA-DSBs. DNA-DSBs with strong cytotoxicity could be repaired in normal cells by the homologous recombination (HR) repair pathway mediated by BRCA gene. However, in the cells with BRCA mutation, DNA-DSBs could not be repaired, or just be repaired by non homologous end joining pathway (NHEJ) alternatively due to lack of HR, thereby greatly increasing the probability of cell death [10, 11].

This study explores these two key DNA repair genes of PARP-1 and BRCA by natural BRCA mutant breast cancer cells to study the role of two genes in DNA damage repair, apoptosis, and radiosensitivity after irradiation. We found that the DNA damage, cell apoptosis, and radiosensitivity of BRCA mutant cells MDA-MB-436 after irradiation increased significantly compared with BRCA non-mutant cells MDA-MB-231, which verified the important role of BRCA gene in DNA damage repair. However, the PARP-1 inhibitor 3-AB combined with irradiation could further cause DNA damage, cell apoptosis, and increasing radiosensitivity of the two kinds of cells, especially of BRCA mutant cells MDA-MB-436. This phenomenon suggests that PARP-1 inhibitors and ionizing radiation have a synergistic effect. When the DNA-SSBs and DNA-DSBs repair pathways are blocked simultaneously, both of SSB and DSB cannot be repaired timely, leading to increased DNA damage significantly, cell apoptosis and final increased radiosensitivity.

Therefore, PARP-1 inhibitors can not only be used as a general radiotherapy sensitizing agent, but also can be used as specific drugs

for certain tumors with suppressor gene mutations, such as BRCA mutation breast cancer, which will greatly increase the treatment effects [6, 12]. The increase of DNA damage, cell apoptosis and radiosensitivity of BRCA mutation breast cancer cells in our study provide the theoretical guidance for improving the radiotherapy efficacy of BRCA mutant breast cancer by using PARP-1 inhibitors. However, there are still some problems, such as how to select better concentration of PARP-1 inhibitor to avoid adverse drug reactions. To solve these problems, we need further in-depth study of specific regulatory mechanisms of PARP1 inhibitors in BRCA mutated breast cancer cells. The results of numerous in vitro studies also need thorough animal experiments and clinical trials to further verify.

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Disclosure of conflict of interest

None.

Address correspondence to: Ling Li, Department of Radiation Oncology, Affiliated Tumor Hospital of Guangxi Medical University, Nanning 530021, Guangxi Autonomous Region, China. E-mail: lilingmoon99@163.com

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