

Increasing radiosensitivity by the combined inhibition of PARP1 and PI3K in BRCA1-mutated triple negative breast cancer

J. Zhou¹, L.Y. Tang², X.H. Zhang³, J. Wang⁴, L.C. Yang^{5*}, S.G. Wu^{4*}

¹Department of Obstetrics and Gynecology, the First Affiliated Hospital of Xiamen University, Xiamen 361003, People's Republic of China

²Eye Institute of Xiamen University, Fujian Provincial Key Laboratory of Ophthalmology and Visual Science, Medical College, Xiamen University, Xiamen 361005, People's Republic of China

³Department of Pharmacy, Xiamen University Hospital, Xiamen 361005, People's Republic of China

⁴Department of Radiation Oncology, Xiamen Cancer Hospital, the First Affiliated Hospital of Xiamen University, Xiamen 361003, People's Republic of China

⁵Xiamen Key Laboratory of Chiral Drugs, Medical College, Xiamen University, Xiamen 361005, People's Republic of China

ABSTRACT

► Original article

*Corresponding authors:

L.C. Yang, Ph.D.,

S.G. Wu Ph.D.,

E-mail:

yanglc116@xmu.edu.cn

unowu12345@hotmail.com

Revised: April 2019

Accepted: May 2019

Int. J. Radiat. Res., April 2020;
18(2): 283-293

DOI: 10.18869/acadpub.ijrr.18.2.283

J. Zhou and L.Y. Tang contributed equally to this work.

Background: To evaluate the radiosensitizing effect of co-targeting of poly (ADP-ribose) polymerase-1 (PARP1) (AZD2461) and phosphoinositide-3-kinase (PI3K) (LY294002) in breast cancer 1, early onset gene (BRCA1)-mutated triple negative breast cancer (TNBC) treated in vitro. **Materials and Methods:** We established HCC1937-PARP1 cells by transfection. Cell proliferation, cell viability, cell cycle, and cell apoptosis were measured and analyzed. Western blotting and quantitative real-time polymerase chain reaction assays were performed. **Results:** The cell viability of HCC1937 and HCC1937-PARP1 cells was significantly decreased under 5 Gy of irradiation. Cell apoptosis was remarkably increased by irradiation, whereas overexpression of PARP1 resulted in substantial resistance to the radiation-induced changes. Combined inhibition of PARP1 and PI3K enhanced radiation-induced apoptosis and significantly inhibited cell proliferation compared with single-agent treatment. The PI3K inhibitor induced changes in the cell cycle distribution, but the PARP1 inhibitor did not. The expression levels of LKB1, PHLPP and INPP4B increased after combined inhibition of PARP1 or PI3K compared with irradiation alone. Moreover, combined inhibition of PARP1 and PI3K resulted in increased expression of INPP4B when compared with that induced by single-agent treatment. **Conclusion:** Combined inhibition of PARP1 and PI3K might be an effective therapeutic strategy to enhance radiosensitivity in BRCA1-mutated TNBC.

Keywords: Breast cancer, radiation, BRCA1, PARP1, PI3K.

INTRODUCTION

Triple negative breast cancer (TNBC) is a special subtype of breast cancer with highly aggressive characteristics, which is associated with a higher rate of early locoregional and distant recurrence ⁽¹⁾. Radiotherapy is an important part of local treatment in patients with breast cancer, including those with TNBC.

However, studies by our group and others have found that the locoregional recurrence rates after postoperative radiotherapy were significantly higher in the TNBC subtype than non-TNBC subtypes ⁽²⁻⁴⁾, which suggested an underlying radioresistance of TNBC ⁽⁵⁾. Locoregional recurrence after postoperative radiotherapy appears to be related to the intrinsic radiotherapy sensitivity of the tumor

cells, which might be caused by abnormal activation and inactivation of oncogenes and tumor suppressor genes. The breast cancer 1, early onset gene (BRCA1) is a tumor suppressor gene that seems to be associated with the TNBC subtype. Approximately 30% of patient with TNBC are BRCA1 mutation carriers, while 90% of BRCA1-mutated patients have the TNBC subtype ⁽⁶⁾.

In light of the significant prevalence of BRCA1 mutations in patients with TNBC, there is a growing interest in the interplay between the loss of the DNA repair function caused by BRCA1 mutations and that caused by the pharmacological inhibition of poly(ADP-ribose) polymerase (PARP), a key enzyme that is activated when a DNA strand breaks and is involved in DNA single-strand break (SSB) repair after radiation (RT) ⁽⁷⁻⁹⁾. PARP1 is the most abundant member of the PARP enzyme family and is involved in base-excision repair (BER) ⁽¹⁰⁾. BRCA1 plays an important role in the homologous recombination (HR) repair of DNA double strands ⁽¹¹⁾. In addition, PARP1 has emerged as a promising therapeutic target for cancers with BRCA1 mutations *via synthetic lethality* ^(9,12). Therefore, targeting PARP1 in BRCA1-mutated cells inhibits the BER machinery that contributes to DNA repair, thereby inducing apoptosis in these cells ⁽¹³⁻¹⁵⁾. Previous studies have demonstrated that BRCA1-mutated cells are hypersensitive to PARP inhibitors and consequently, apoptosis occurs because of increased genomic instability. Preclinical studies have shown that PARP1 inhibition improves the radiosensitivity of BRCA1-mutated breast cancer cells ⁽¹³⁻¹⁶⁾.

The phosphoinositide-3-kinase/AKT (PI3K/AKT) pathway is one of the most commonly deregulated cancer-associated signaling pathways. PI3K/AKT promotes the proliferation and survival of tumor cells, but also regulates steady-state levels of HR ⁽¹⁷⁻¹⁹⁾. Several studies have shown that activation of the PI3K/AKT signaling pathway in breast cancer will lead to abnormal functions of genes and may increase the resistance of tumor cells to chemotherapy and RT ⁽²⁰⁾. Phosphorylation of AKT may also be activated in BRCA1-deficient TNBC cells

compared with non-BRCA1 TNBC cells, and inhibiting the PI3K/AKT signaling pathway might increase the anti-tumor effect of BRCA1-mutated TNBC cells ^(21,22). Furthermore, co-targeting PI3K might sensitize BRCA1-mutated TNBC to PARP inhibition ⁽²³⁾.

PARP1 inhibition has been confirmed to decrease the risk of disease progression or death among patients with metastatic breast cancer who are also BRCA mutation carriers ⁽²⁴⁾. In addition, AKT inhibitors might also increase the progression-free survival of metastatic TNBC ⁽²⁵⁾. However, few studies have assessed the radiotherapy effect on co-targeting of PARP1 and PI3K/AKT in BRCA1-mutated TNBC ⁽²⁶⁾. Despite the effects of multimodality therapy for patients with breast cancer, sustained locoregional control remains an important issue for certain patients, especially those with TNBC. Based on the abovementioned findings, we postulated that the combined inhibition of PARP1 and PI3K/AKT would sensitize BRCA1-mutated TNBC to RT. To further support the potential for the clinical translation of this work, we investigated the potential radiosensitizing effect of combined inhibition of PARP1 and PI3K/AKT in BRCA1-mutated TNBC cells treated *in vitro*.

MATERIALS AND METHODS

Materials

The following antibodies were used in this study: Rabbit anti-Ki67⁺ ((Active Motif Corp, California, USA).); Rabbit anti-Inositol polyphosphate-4-phosphatase (INPP4B), anti-PH domain and Leucine rich repeat Protein Phosphatases (PHLPP), anti-γH2aX, anti-LKB1, anti-phosphorylated LKB1, anti-phosphorylated AKT, and HRP-conjugated mouse anti-rabbit β-tubulin (Cell Signaling Technology Group, Inc., Boston, USA); HRP-conjugated rabbit anti-goat IgG (Wuhan Boster Biological Technology Group, Wuhan, China); and Rabbit anti-AKT (Proteintech Corp, Wuhan, China.). Other materials used were a Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA); fetal bovine serum (FBS, Invitrogen; a

Annexin V-PE/7-AAD Apoptosis Detection Kit (YEASEN, Shanghai, China); a Cell Counting Kit-8 was obtained from BBI Life Sciences; a propidium iodide (PI) staining kit (Sangon Biotech, Shanghai, China). The inhibitors used were a poly (ADP-ribose) polymerase-1 (PARP1) inhibitor AZD2461 (Selleckchem, Houston, USA); a phosphoinositide-3-kinase (PI3K) inhibitor LY294002 (Selleckchem).

Cell Culture

HCC1937-PARP1 cells were established by transfecting with human HCC1937 cells by a plasmid, which overexpressed human PARP1, were cultured in six-well plates with Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS in an incubator at 37 °C with 95% humidity and 5% CO₂. Cells were cultured for 12 hours before treatment with X-ray irradiation (radiation dose = 5 Gy). After irradiation, the cells were treated with 200 μM AZD2461 (PARP1 inhibitor), or 200 μM LY294002 (PI3K inhibitor), or 200 μM AZD2461 plus LY294002, respectively. After treatment for 96 hours, the cells were used for subsequent experiments.

Western Blotting Assay

Total cellular proteins were extracted from of HCC1937-PARP1 cells using cold Radioimmunoprecipitation assay (RIPA) buffer containing a proteinase inhibitor cocktail (Beyotime Institute of Biotechnology, China) and quantified using a BCA Protein Assay Kit (ThermoFisher Scientific). The standard western blotting protocol was applied. The following antibodies were used: anti-INPP4B (CST, catalog no. 14543, 1:1000), anti-PHLPP (CST, catalog no. 2947s, 1:1000), anti-γH2aX (CST, catalog no. 9718s, 1:1000), anti-Ki67⁺ (Active. Motif, catalog no. 39799, 1:500); anti-AKT (Proteintech, catalog no. 10176-2-AP, 1:1000); anti-phosphorylated AKT (CST, catalog no. 9271, 1:1000); anti-LKB1 (CST, catalog no. 3050, 1:1000); anti-phosphorylated LKB1 (CST, catalog no. 3055, 1:1000); anti-β-tubulin (CST, catalog no. 2128, 1:5000). After incubation with the appropriate secondary antibodies, the immunoreactive protein bands were visualized using an enhanced chemiluminescence reagent

(Thermo, catalog no. NCI5079) and the images were recorded using a transilluminator (ChemiDoc XRS System; BioRad, Philadelphia, PA, USA).

RNA Isolation and Quantitative Real-Time PCR

We performed quantitative real-time RT-PCR (qRT-PCR) as previously described. Total RNA was extracted from HCC1937-PARP1 cells using the TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using a Transcriptor First-Strand cDNA synthesis kit (Fermentas, catalog no. #K1622). Quantitative real-time PCR was performed using the StepOne Real-Time PCR System with a SYBR Premix Ex TaqKit (Fermentas, catalog no. #K0242). The primer sequences are shown in table 1. The amplification program included an initial denaturation step at 95 °C for 10 minutes; followed by 40 cycles of denaturation at 95 °C for 10 seconds and annealing and extension at 60 °C for 30 seconds; after which a melt curve analysis was conducted to check the amplification specificity. The results of the qRT-PCR were analyzed by the comparative threshold cycle (Ct) method and were normalized to the expression of *GAPDH*.

Table 1. Human Primer Sequences Used for Quantitative Real-Time PCR.

Gene	Sense Primer	Antisense Primer
<i>INPP4b</i>	CCAGAAGACT CCAAATGAACCG	ACGGGGTGGGA TTACGGGAGA
<i>PHLPP</i>	ATCCTATGTGG GACCTGCCT	AGTTCATTAAG CCCCCTGGC
<i>LKB1</i>	GACCTGCTGAA AGGG ATGCT	TGTCCATTGT GACTGGCCTC
<i>Ki 67⁺</i>	ACGCCTGGTTAC TATCAAAAGG	CAGACCCATTTA CTTGTGTTGGA
<i>γH2aX</i>	AGAAGACGCG AATCATCCCC	GGCGCTGGTCTTCTTGG
<i>GAPDH</i>	CTGGGGCTACAC TGAGCACC	AAGTGGTCGTTG AGGGCAATG

Cell Apoptosis

Cell apoptosis was determined using an Annexin V-PE/7-AAD apoptosis detection Kit (YEASEN, catalog no. 40310) following the manufacturer's protocol. HCC1937-PARP1 cells were cultured on 6-well plates for 12 hours

before treatment with X-ray irradiation (radiation dose = 5 Gy). The medium was replaced by conditioned medium containing with the same concentration of AZD2461 or LY294002 or AZD2461 plus LY294002 respectively. After treatment for 96 hours, the cells were collected after trypsin digestion, followed by incubation for 30 minutes at 37 °C in the dark. Cell apoptosis was then detected using flow cytometry.

Cell Viability Assay

Cell viability was examined using a Cell Counting Kit-8 (BBI Life Sciences, catalog no. E606335-0500) according to the manufacturer's instructions. HCC1937-PARP1 cells were cultured on 96-well plates for 12 hours, and then separately treated with X-ray irradiation (radiation dose = 5 Gy) for 48 hours. The medium was replaced by CCK-8 constituted in culture media, followed by incubation for 2 hours at 37 °C in the dark. The absorbance of the solution at 450 nm was then determined directly using a Bio Tek ELX800 microplate reader (Bio Tek Instruments, Winooski, VT, USA).

Cell Cycle Assay

The proportions of cells at the various stages of the cell cycle were determined using a PI Staining Kit (Sangon Biotech, catalog no. E607306) following the manufacturer's protocol. HCC1937-PARP1 cells were cultured on 6-well plates for 12 hours before treatment with X-ray irradiation (radiation dose = 5 Gy). The medium was replaced by conditioned medium containing with the same concentration of AZD2461, LY294002 or their combination,

respectively. After treatment for 96 hours, the cells were collected after trypsin digestion, and fixed in 75% ethanol overnight. After removing the alcohol, the suspended cells were added with 2.5 µL of RNase, followed by incubation for 30 minutes at 37 °C, after which 50 µL PI solution was added. The proportions of cells at the various stages of the cell cycle were detected using flow cytometry.

Statistical Analysis

The experiments described above were repeated three times. One-way analysis of variance test (ANOVA) with Tukey's post hoc test was conducted to analyze the data among three or more groups. Student's t-test was conducted to analyze the data when comparing two groups. Differences were considered significant at $p < 0.05$.

RESULTS

Comparison of Cellular Responses to X-ray irradiation and PARP1

We first applied X-ray irradiation to cultured HCC1937 and HCC1937-PARP1 cells, and measured the cell viability and apoptosis using CCK-8 and Annexin V-PE/7-AAD assays, respectively. The viability of HCC1937 and HCC1937-PARP1 cells greatly decreased under 5 Gy irradiation; however, overexpression of PARP1 restored cell viability to control levels (figure 1A). Cell apoptosis increased markedly after irradiation and overexpression of PARP1 could substantially resistance this increase in apoptosis (figure 1B).

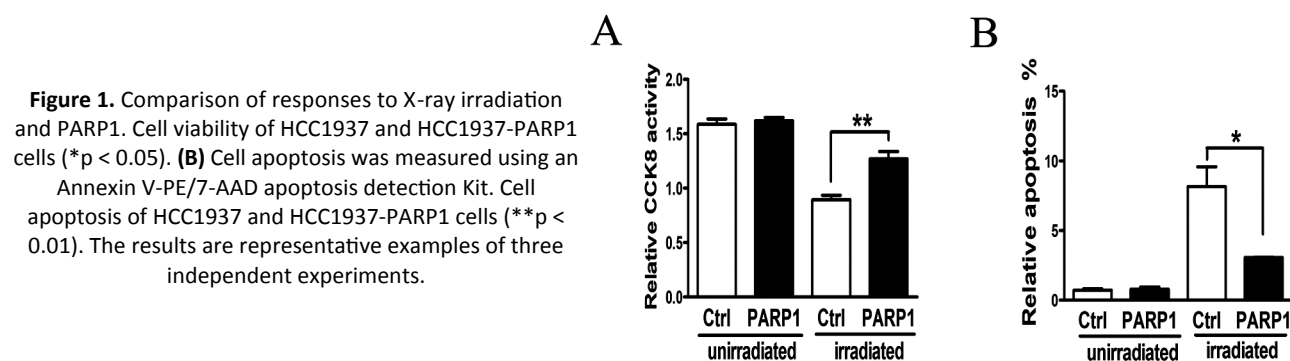


Figure 1. Comparison of responses to X-ray irradiation and PARP1. **(A)** Cell viability of HCC1937 and HCC1937-PARP1 cells (* $p < 0.05$). **(B)** Cell apoptosis was measured using an Annexin V-PE/7-AAD apoptosis detection Kit. Cell apoptosis of HCC1937 and HCC1937-PARP1 cells (** $p < 0.01$). The results are representative examples of three independent experiments.

AZD2461 and LY294002 promoted HCC1937-PARP1 cell apoptosis

To evaluate the effect of PARP1 inhibitor AZD2461 and PI3K inhibitor LY294002 on cell apoptosis of HCC1937-PARP1 cell, we detected apoptosis in HCC1937-PARP1 cells treated with AZD2461 and LY294002 after irradiation, either alone or in combination, for 96 hours. Compared with that in the RT group, the level of apoptosis increased in the AZD+RT, LY+RT, and AZD+LY+RT groups. Furthermore, the combined use of the PARP1 and PI3K inhibitors enhanced radiation-induced apoptosis compared with that induced by each agent separately (figure 2A). The results indicated that a combination of PARP1 and / or PI3K inhibition and RT increased apoptosis compared with RT alone.

AZD2461 and LY294002 inhibited HCC1937-PARP1 cell proliferation

To evaluate the effect of the PARP1 inhibitor

AZD2461 and PI3K inhibitor LY294002 on cell proliferation of HCC1937-PARP1 cell, we detected the clone formation ability of the cells and the expression of Ki67⁺, a marker of proliferation. The results showed that the clone formation ability of the cells greatly decreased after receiving irradiation compared with the untreated control group (figure 3A). Compare with that in the RT group, cell proliferation was markedly reduced in the AZD+RT, LY+RT, or combination treatment. The combined use of PARP1 and PI3K inhibition greatly inhibited cell proliferation compared with that induced by each agent alone. The mRNA (figure 3B) and protein (figure 3C-D) levels of Ki67⁺ were markedly decreased in the AZD+RT and LY+RT group compared with those in the RT alone group. Moreover, the combined use of PARP1 and PI3K inhibition reduced Ki67⁺ expression to a greater extent compared with that induced by each agent alone.

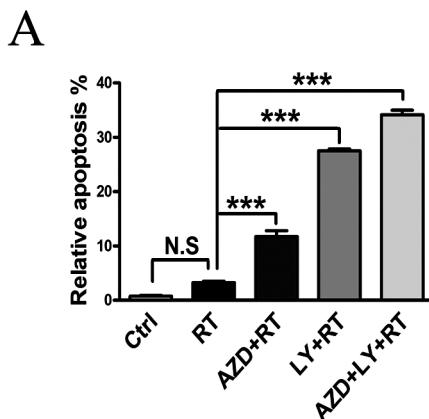


Figure 2. AZD2461 and LY294002 promoted HCC1937-PARP1 cell apoptosis.

Cell apoptosis was measured in the groups comprising the control, irradiation only, and irradiation plus treatment with AZD2461 alone, LY294002 alone, and AZD2461 plus LY294002 (***p* < 0.001). The results are representative examples of three independent experiments.

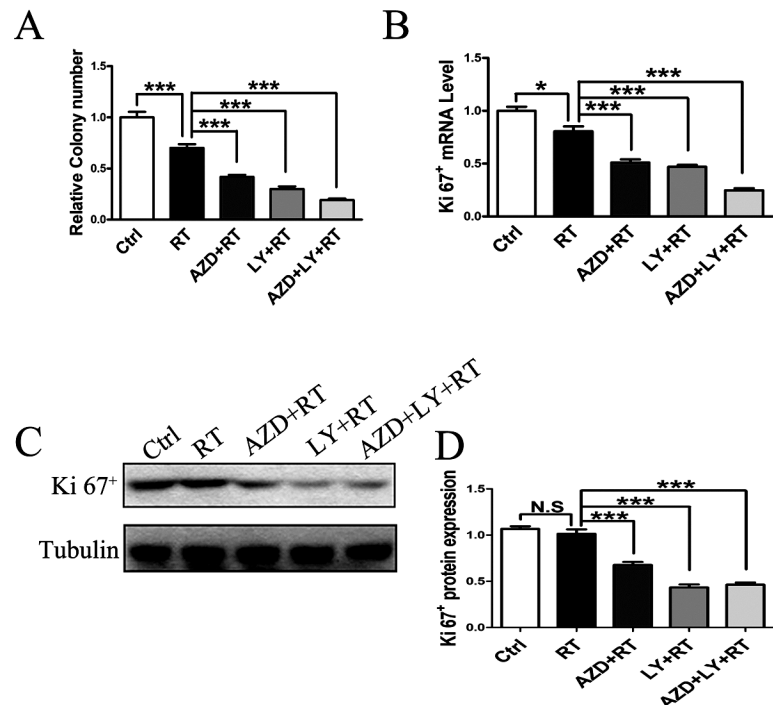


Figure 3. AZD2461 and LY294002 inhibited HCC1937-PARP1 cell proliferation.

(A) The number of colonies was measured in the groups comprising the control, irradiation only, and irradiation plus treatment with AZD2461 alone, LY294002 alone, and AZD2461 plus LY294002 (*n* = 5 for each group) (**p* < 0.05, ****p* < 0.001). (C) Representative western blotting images of Ki67⁺. (D) The statistical analysis of the western blotting results for Ki67⁺ (***p* < 0.001). The results are representative examples of three independent experiments.

The PI3K inhibitor, but not the PARP1 inhibitor, induced changes in the cell cycle distribution

To evaluate the effect of AZD2461 and LY294002 on the cell cycle in HCC1937-PARP1 cell, PI staining followed by flow cytometry was used. Cells in the G2 phase of the cell cycle are radiosensitive. We found that the number of cells in the G2 phase was greatly increased in RT group compared with that in the control cells, while it was reduced in the AZD+RT group and increased in the LY+RT group compared with that in the RT group (figure 4A). The combined inhibitor treatment could greatly reduced the number of cells in all phases of the cell cycle. This indicated that the PI3K/AKT signal pathway might be associated with impaired HR.

The formation of γ H2aX (the phosphorylated form of H2A histone family member X) is a marker of DNA double-strand breaks (DSBs) induced by RT. We found that the mRNA (figure 4B) expression levels of *H8aX* were remarkable increased in the LY+RT and combination treatment groups compared with that in the RT alone group. However, there was no effect on the protein levels of γ H2aX in cells treated with a combination of PARP1 and/or PI3K inhibition and RT compared with that in the RT alone group (figure 4C-D). This result indicated that there was no significant correlation between treatment-induced changes in the cell cycle distribution and PARP1-mediated radiosensitivity in BRCA1-mutated TNBC cells.

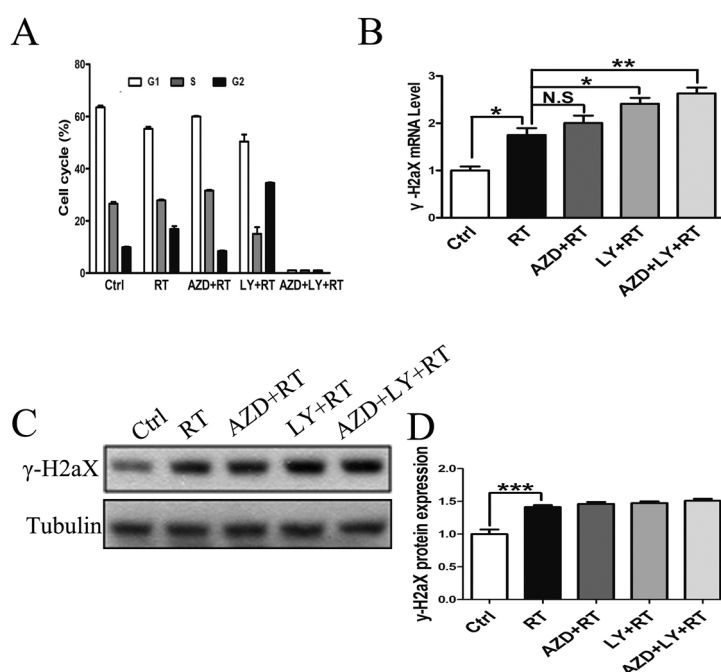


Figure 4. The PI3K inhibitor, but not the PARP1 inhibitor, induced changes in cell cycle distribution. **(A)** The cell cycle distribution was measured in the comprising the control, irradiation only, and irradiation plus treatment with AZD2461 alone, LY294002 alone, and AZD2461 plus LY294002. **(B)** qRT-PCR of H2aX expression was measured in the groups mentioned in (A) (* $p < 0.05$, ** $p < 0.01$). **(C)** Representative western blotting images of γ H2aX. **(D)** The statistical analysis of the western blotting results for γ H2aX (*** $p < 0.001$). The results are representative examples of three independent experiments.

Overexpression of INPP4B inhibited the PI3K/AKT signaling pathway

To investigate whether the increased radiosensitization in BRCA1-mutated TNBC cells was associated with suppression of the PI3K/AKT signaling pathway, levels of the AKT protein

and its phosphorylated (active) form were compared in among the groups. To further substantiate the association between suppression of PI3K/AKT signaling pathway and increased radiosensitization in BRCA1-mutated TNBC cells, we determined if the expressions

levels of PHLPP, LKB1, and INPP4B were altered, as they are important negative regulators of PI3K/AKT signaling pathway in cell proliferation and malignant transformation. The mRNA and protein expression levels of PHLPP, LKB1, and INPP4B were increased after the combined inhibition of PARP1 and PI3K compared with those induced by RT alone (figure 5A [a-c], 5B [a-d]). Moreover, the combined inhibition of PARP1 and PI3K resulted in an increasing expression of INPP4B when compared with that induced by each agent alone. The level of the

phosphorylated AKT protein was greatly reduced following treatment with AZD+RT, LY+RT, or the combination treatment compared with that in the RT alone group (figure 5B[e]). These data support the contention that TNBC is associated with loss of heterozygosity in INPP4B, resulting in increased levels of phosphorylated AKT and activation of the PI3K/AKT signaling pathway, which leads to cell proliferation, and thereby inhibiting malignant transformation of cells.

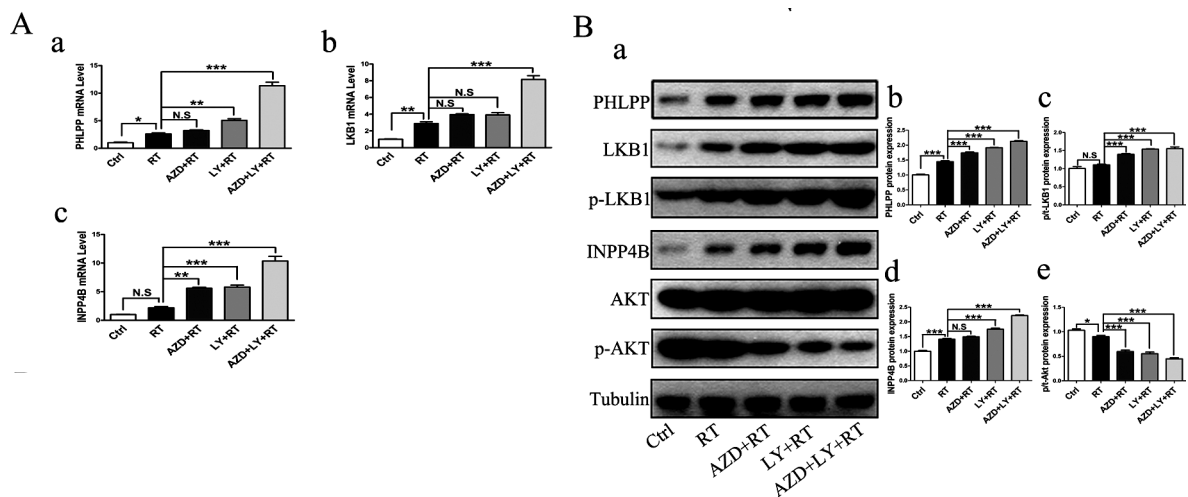


Figure 5. Overexpression of INPP4B inhibited the PI3K/AKT signaling pathway. **(A)** qRT-PCR for PHLPP **(a)**, LKB1 **(b)** and INPP4B **(c)** expression was measured in the groups comprising the control, irradiation only, and irradiation plus treatment with AZD2461 alone, LY294002 alone, and AZD2461 plus LY294002 (** $p < 0.01$, *** $p < 0.001$). **(B)** **(a)** Representative western blotting results for PHLPP, LKB1, and INPP4B. **(b-e)** The statistical analysis of the western blotting results for PHLPP, LKB1, and INPP4B (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The results are representative examples of three independent experiments.

DISCUSSION

The current study investigated the potential radiosensitizing effect of the combined inhibition of PARP1 and PI3K/AKT in BRCA1-mutated TNBC cells treated *in vitro*. Our results demonstrated that co-targeting PARP1 and PI3K/AKT increased the radiosensitization in BRCA1-mutated TNBC cells. Our findings provide evidence that PARP1 and PI3K/AKT inhibition could serve as a viable means of radiosensitizing BRCA1-mutated TNBC cells.

PARP proteins have been studied for decades and are known to play important roles in a variety of cellular functions ⁽²⁷⁾. Targeting synthetic lethality by PARP inhibition might be

very effective as a single agent therapy in patients whose cancers have either somatic or germline defects in DNA damage and repair genes, or defects in genes involved in PTEN signaling. Specific defects in DNA repair pathways also appear to increase the radiosensitizing effects of PARP inhibition ⁽⁹⁾. Our results indicated that a combination of PARP1 inhibition and RT reduced clonogenic survival and increased apoptosis compared with RT alone, which were similar to previous studies in lung, pancreatic, and head and neck cancer ⁽²⁸⁻³⁰⁾. PARP1 inhibition has been confirmed to decrease the risk of disease progression or death among patients with metastatic breast cancer with a germline BRCA mutation ⁽²⁴⁾.

However, to the best of our knowledge, there are no clinical results that assess the radiosensitizing effects of PARP1 inhibition in breast cancer with locoregional or distant recurrence. In TNBC, there is a higher incidence of locoregional recurrence and distant metastasis, such as in the brain or lung, compared with other subtypes. In addition, there is a lack of specific treatment targets, such as endocrine therapy and anti-human epidermal growth factor receptor 2 treatment. To decrease locoregional recurrence and to treat distant metastasis, such as brain metastasis or other oligometastasis, PARP1 represents an interesting target to enhance the effect of RT on BRCA1-mutated TNBC.

The formation of γ H2aX is the marker of DNA DSBs induced by RT. PARP1 is a multi-functional protein that can regulate a variety of cellular pathways, including DNA SSB repair, HR, non-homologous end joining (NHEJ), and transcriptional regulation⁽³¹⁻³³⁾, while the *BRCA1* gene plays a key role in DNA HR. Our study found that there was no effect on the protein level of γ H2aX in the AZD+RT, LY+RT, or combination treatment groups, while increased *H2aX* gene expression was observed in the LY+RT and combination treatment groups compared with that induced by RT alone. In addition, we found that the number of cells in the G2 phase (the radiosensitive phase of the cell cycle) was significantly increased in the LY+RT group, while there was no effect on the cell cycle distribution in AZD+RT cells, which suggested that the radiosensitizing effect of the PARP1 inhibitor was not related to cell cycle changes. Our results were similar to those reported by Feng *et al.*⁽¹⁶⁾, in which the expression of γ H2aX was not associated with radiosensitivity in AZD+RT cells, and there was also no significant correlation between treatment-induced changes in cell cycle distribution and PARP1-mediated radiosensitivity in BRCA1-mutated TNBC cells. Given the diversity of cellular pathways affected by PARP1, it is unlikely that the molecular mechanism of the radiosensitization effects of PARP inhibitors reflects a singular function of PARP1 and is more

likely to be multifaceted.

PI3K suppression has been shown to impair HR in the cellular DNA damage response pathway^(18,19). Therefore, we further explored the effect of combined inhibition of PARP1 and PI3K on radiosensitivity. HR is an error-prone process that can only occur during the S and G2 phases, and requires sister chromatids as templates to repair the damaged DNA^(9,34). Our study further confirmed that the PI3K inhibitor could significantly increase the number of cells in the G2 phase after RT, suggesting a role of the PI3K/AKT signal pathway in impaired HR. In addition, the combined use of PARP1 and PI3K inhibition enhanced radiation-induced apoptosis and inhibited cell proliferation compared with those induced by each agent alone. A previous study by Jang *et al.*⁽²⁶⁾ also found that co-targeting the PI3K pathway (using a pyridinylfuranopyrimidine inhibitor) and PARP1 enhanced RT-induced cell death in BRCA1-mutated TNBC cells and xenografts. Therefore, based on the abovementioned findings, we suggest that additional PI3K inhibition combined with PARP inhibition in BRCA1-mutated TNBC cells may enhance the response to RT.

INPP4B, PHLPP, and LKB1 are important negative regulators of the PI3K/AKT signaling pathway in cell proliferation and malignant transformation⁽³⁵⁻³⁹⁾. Therefore, in the present study, we analyzed the expression of three above genes in *each* treatment group. The results showed a significant effect on the expression of *INPP0B*, *PHLPP*, and *LKB1* after treatment with RT and the two inhibitors, alone or in combination, compared with RT alone. Combined inhibition of PARP1 and PI3K resulted in increased *INPP0B*, *PHLPP*, and *LKB1* gene expression compared with that induced by each agent alone.

INPP4B is a tumor suppressor that maintains the phospholipid balance by inhibiting the activation of PI3K/AKT signaling pathway, thereby inhibiting malignant transformation of cells⁽³⁶⁾. TNBC is associated with loss of a heterozygosity in *INPP0B*, resulting in increased mRNA levels of phosphorylated AKT and activation of the PI3K/AKT signaling pathway,

which leads to cell proliferation, migration, and angiogenesis⁽³⁷⁾. Ding *et al.*⁽⁴⁰⁾ found that the combination of a PARP inhibitor and *INPP0B* gene transfection had a synergistic therapeutic effect on castration therapy-resistant prostate cancer cells. Sun *et al.*⁽⁴¹⁾ also found that overexpression of *INPP0B* enhanced PARP1 inhibitor-induced proliferation inhibition in TNBC cells. Therefore, based on the abovementioned findings and the results of the present study, the suppression of PI3K/AKT signaling pathway caused by *INPP0B* overexpression might contribute to the increase radiosensitivity induced by combined therapy with the PARP1 inhibitor.

The limitation of our study is that the effects were shown only in *vitro* studies, which limited our results extend to clinical significance. Since the microenvironment is important for radiosensitivity, more in *vivo* studies are need to confirm our results.

Our results suggest that combinations of targeted agents such as PAPR1 and PI3K inhibitors might be an effective therapeutic strategy to enhance radiosensitivity in BRCA1-mutated TNBC. The development of co-targeting PARP1 and PI3K signaling with RT as a strategy to treat BRCA1-mutated TNBC will require careful preclinical studies.

ACKNOWLEDGMENTS

This work was partly supported by the National Natural Science Foundation of China (No. 81402527), Natural Science Foundation of Fujian Province (No. 2016J01635), and the Science and Technology Planning Projects of Xiamen Science & Technology Bureau (No. 3502Z20174070).

Conflicts of interest: Declared none.

REFERENCES

1. Foulkes WD, Smith IE, Reis-Filho JS (2010) Triple-negative breast cancer. *N Engl J Med*, **36**: 1938-1948.
2. Hattangadi-Gluth JA, Wo JY, Nguyen PL, Abi Raad RF, Sreedhara M, Niemierko A, Freer PE, Georgian-Smith D, Bellon JR, Wong JS, Smith BL, Harris JR, Taghian AG (2012) Basal subtype of invasive breast cancer is associated with a higher risk of true recurrence after conventional breast-conserving therapy. *Int J Radiat Oncol Biol Phys*, **82**: 1185-1191.
3. Wu SG, He ZY, Li Q, Li FY, Lin Q, Lin HX, Guan XX (2012) Predictive value of breast cancer molecular subtypes in Chinese patients with four or more positive nodes after postmastectomy radiotherapy. *Breast*, **21**: 657-661.
4. Kyndi M, Sørensen FB, Knudsen H, Overgaard M, Nielsen HM, Overgaard J; Danish Breast Cancer Cooperative Group (2008) Estrogen receptor, progesterone receptor, HER-2, and response to postmastectomy radiotherapy in high-risk breast cancer: the Danish Breast Cancer Cooperative Group. *J Clin Oncol*, **26**: 1419-1426.
5. Eiermann W, Vallis KA (2012) Locoregional treatments for triple-negative breast cancer. *Ann Oncol*, **23** Suppl 6: vi30-34.
6. Cleator S, Heller W, Coombes RC (2007) Triple-negative breast cancer: therapeutic options. *Lancet Oncol*, **8**: 235-244.
7. Rouleau M, Patel A, Hendzel MJ, Kaufmann SH, Poirier GG (2010) PARP inhibition: PARP1 and beyond. *Nat Rev Cancer*, **10**: 293-301.
8. Annunziata CM, Bates SE (2010) PARP inhibitors in BRCA1/BRCA2 germline mutation carriers with ovarian and breast cancer. *F1000 Biol Rep*, **2**: pii:10.
9. Chalmers AJ, Lakshman M, Chan N, Bristow RG (2010) Poly (ADP-ribose) polymerase inhibition as a model for synthetic lethality in developing radiation oncology targets. *Semin Radiat Oncol*, **20**: 274-281.
10. Dantzer F, Schreiber V, Niedergang C, Trucco C, Flatter E, De La Rubia G, Oliver J, Rolli V, Ménissier-de Murcia J, de Murcia G (1999) Involvement of poly(ADP-ribose) polymerase in base excision repair. *Biochimie*, **81**: 69-75.
11. Roy R, Chun J, Powell SN (2011) BRCA1 and BRCA2: different roles in a common pathway of genome protection. *Nat Rev Cancer*, **12**: 68-78.
12. Kaelin WG Jr (2005) The concept of synthetic lethality in the context of anticancer therapy. *Nat Rev Cancer*, **5**: 689-698.
13. Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, Santarosa M, Dillon KJ, Hickson I, Knights C, Martin NM, Jackson SP, Smith GC, Ashworth A (2005) Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*, **434**: 917-921.
14. Rottenberg S, Jaspers JE, Kersbergen A, van der Burg E, Nygren AO, Zander SA, Derksen PW, de Bruin M, Zevenhoven J, Lau A, Boulter R, Cranston A, O'Connor MJ, Martin NM, Borst P, Jonkers J (2008) High sensitivity of BRCA1-deficient mammary tumors to the PARP inhibitor AZD2281 alone and in combination with platinum drugs. *Proc Natl Acad Sci USA*, **105**: 17079-17084.
15. Clark CC, Weitzel JN, O'Connor TR (2012) Enhancement of synthetic lethality via combinations of ABT-888, a PARP inhibitor, and carboplatin in vitro and in vivo using BRCA1 and BRCA2 isogenic models. *Mol Cancer Ther*, **11**: 1948-

- 1958.
16. Feng FY, Speers C, Liu M, Jackson WC, Moon D, Rinkinen J, Wilder-Romans K, Jagsi R, Pierce LJ (2014) Targeted radiosensitization with PARP1 inhibition: optimization of therapy and identification of biomarkers of response in breast cancer. *Breast Cancer Res Treat*, **147**: 81-94.
17. López-Knowles E, O'Toole SA, McNeil CM, Millar EK, Qiu MR, Crea P, Daly RJ, Musgrove EA, Sutherland RL (2010) PI3K pathway activation in breast cancer is associated with the basal-like phenotype and cancer-specific mortality. *Int J Cancer*, **126**: 1121-1131.
18. Kumar A, Fernandez-Capetillo O, Carrera AC (2010) Nuclear phosphoinositide 3-kinase beta controls double-strand break DNA repair. *Proc Natl Acad Sci USA*, **107**: 7491-7496.
19. Toulany M, Lee KJ, Fattah KR, Lin YF, Fehrenbacher B, Schaller M, Chen BP, Chen DJ, Rodemann HP (2012) Akt promotes post-irradiation survival of human tumor cells through initiation, progression, and termination of DNA-PKcs-dependent DNA double-strand break repair. *Mol Cancer Res*, **10**: 945-957.
20. Ginestier C, Liu S, Diebel ME, Korkaya H, Luo M, Brown M, Wicinski J, Cabaud O, Charafe-Jauffret E, Birnbaum D, Guan JL, Dontu G, Wicha MS (2010) CXCR1 blockade selectively targets human breast cancer stem cells in vitro and in xenografts. *J Clin Invest*, **20**: 485-497.
21. Yi YW, Kang HJ, Kim HJ, Hwang JS, Wang A, Bae I (2013) Inhibition of constitutively activated phosphoinositide 3-kinase/AKT pathway enhances antitumor activity of chemotherapeutic agents in breast cancer susceptibility gene 1-defective breast cancer cells. *Mol Carcinog*, **52**: 667-675.
22. Yi YW, Hong W, Kang HJ, Kim HJ, Zhao W, Wang A, Seong YS, Bae I (2013) Inhibition of the PI3K/AKT pathway potentiates cytotoxicity of EGFR kinase inhibitors in triple-negative breast cancer cells. *J Cell Mol Med*, **17**: 648-656.
23. Kimbung S, Biskup E, Johansson I, Aaltonen K, Ottosson-Wadlund A, Gruvberger-Saal S, Cunliffe H, Fadeel B, Loman N, Berglund P, Hedenfalk I (2012) Co-targeting of the PI3K pathway improves the response of BRCA1 deficient breast cancer cells to PARP1 inhibition. *Cancer Lett*, **319**: 232-241.
24. Robson M, Im SA, Senkus E, Xu B, Domchek SM, Masuda N, Delaloge S, Li W, Tung N, Armstrong A, Wu W, Goessl C, Runswick S, Conte P (2017) Olaparib for Metastatic Breast Cancer in Patients with Germline BRCA Mutation. *N Engl J Med*, **377**: 523-533.
25. Kim SB, Dent R, Im SA, Espié M, Blau S, Tan AR, Isakoff SJ, Oliveira M, Saura C, Wongchenko MJ, Kapp AV, Chan WY, Singel SM, Maslyar DJ, Baselga J; LOTUS investigators (2017) Ipatasertib plus paclitaxel versus placebo plus paclitaxel as first-line therapy for metastatic triple-negative breast cancer (LOTUS): a multicentre, randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet Oncol*, **18**: 1360-1372.
26. Jang NY, Kim DH, Cho BJ, Choi EJ, Lee JS, Wu HG, Chie EK, Kim IA (2015) Radiosensitization with combined use of olaparib and PI-103 in triple-negative breast cancer. *BMC Cancer*, **15**: 89.
27. D'Amours D, Desnoyers S, D'Silva I, Poirier GG (1999) Poly (ADP-ribose)ylation reactions in the regulation of nuclear functions. *Biochem J*, **342**: 249-268.
28. Khan K, Araki K, Wang D, Li G, Li X, Zhang J, Xu W, Hoover RK, Lauter S, O'Malley B Jr, Lapidus RG, Li D (2010) Head and neck cancer radiosensitization by the novel poly(ADP-ribose) polymerase inhibitor GPI-15427. *Head Neck*, **32**: 381-391.
29. Karnak D, Engelke CG, Parsels LA, Kausar T, Wei D, Robertson JR, Marsh KB, Davis MA, Zhao L, Maybaum J, Lawrence TS, Morgan MA (2014) Combined inhibition of Wee1 and PARP1/2 for radiosensitization in pancreatic cancer. *Clin Cancer Res*, **20**: 5085-5096.
30. Albert JM, Cao C, Kim KW, Willey CD, Geng L, Xiao D, Wang H, Sandler A, Johnson DH, Colevas AD, Low J, Rothenberg ML, Lu B (2007) Inhibition of poly(ADP-ribose) polymerase enhances cell death and improves tumor growth delay in irradiated lung cancer models. *Clin Cancer Res*, **13**: 3033-3042.
31. Mitchell J, Smith GC, Curtin NJ (2009) Poly(ADP-Ribose) polymerase-1 and DNA-dependent protein kinase have equivalent roles in double strand break repair following ionizing radiation. *Int J Radiat Oncol Biol Phys*, **75**: 1520-1527.
32. Schiewer MJ, Goodwin JF, Han S, Brenner JC, Augello MA, Dean JL, Liu F, Planck JL, Ravindranathan P, Chinnaiyan AM, McCue P, Gomella LG, Raj GV, Dicker AP, Brody JR, Pascal JM, Centenera MM, Butler LM, Tilley WD, Feng FY, Knudsen KE (2012) Dual roles of PARP-1 promote cancer growth and progression. *Cancer Discov*, **2**: 1134-1149.
33. Saberi A, Hochegeger H, Szuts D, Lan L, Yasui A, Sale JE, Taniguchi Y, Murakawa Y, Zeng W, Yokomori K, Helleday T, Teraoka H, Arakawa H, Buerstedde JM, Takeda S (2007) RAD18 and poly(ADP-ribose) polymerase independently suppress the access of nonhomologous end joining to double-strand breaks and facilitate homologous recombination-mediated repair. *Mol Cell Biol*, **27**: 2562-2571.
34. Jackson SP (2002) Sensing and repairing DNA double-strand breaks. *Carcinogenesis*, **23**: 687-696.
35. Lopez SM, Hodgson MC, Packianathan C, Bingol-Ozakpinar O, Uras F, Rosen BP, Agoulnik IU (2013) Determinants of the tumor suppressor INPP4B protein and lipid phosphatase activities. *Biochem Biophys Res Commun*, **440**: 277-282.
36. Fedele CG, Ooms LM, Ho M, Vieuxseux J, O'Toole SA, Millar EK, Lopez-Knowles E, Sriratanana A, Gurung R, Baglietto L, Giles GG, Bailey CG, Rasko JE, Shields BJ, Price JT, Majerus PW, Sutherland RL, Tiganis T, McLean CA, Mitchell CA (2010) Inositol polyphosphate 4-phosphatase II regulates PI3K/Akt signaling and is lost in human basal-like breast cancers. *Proc Natl Acad Sci USA*, **107**: 22231-22236.
37. Jung S, Li C, Jeong D, Lee S, Ohk J, Park M, Han S, Duan J, Kim C, Yang Y, Kim KI, Lim JS, Kang YS, Lee MS (2013) Oncogenic function of p34SEI-1 via NEDD4 1 mediated PTEN ubiquitination/degradation and activation of the PI3K/AKT pathway. *Int J Oncol*, **43**: 1587-1595.

38. Newton AC, Trotman LC (2014) Turning off AKT: PHLPP as a drug target. *Annu Rev Pharmacol Toxicol*, **54**: 537-558.
39. Dogliotti G, Kullmann L, Dhumale P, Thiele C, Panichkina O, Mendl G, Houben R, Haferkamp S, Püschel AW, Krahn MP (2017) Membrane-binding and activation of LKB1 by phosphatidic acid is essential for development and tumour suppression. *Nat Commun*, **8**: 15747.
40. Ding H, Sun Y, Hou Y, Li L (2014) Effects of INPP4B gene transfection combined with PARP inhibitor on castration therapy-resistant prostate cancer cell line, PC3. *Urol Oncol*, **32**: 720-726.
41. Sun Y, Ding H, Liu X, Li X, Li L (2014) INPP4B overexpression enhances the antitumor efficacy of PARP inhibitor AG014699 in MDA-MB-231 triple-negative breast cancer cells. *Tumour Biol*, **35**: 4469-4477.

