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Ferulic acid in combination with PARP inhibitor sensitizes breast cancer cells as chemotherapeutic strategy

Young Eun Choi [a], Eunmi Park [b, *]

[a] Department of Radiation Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA
[b] Department of Food and Nutrition, School of Life Science and Nano-Technology, Hannam University, 461-6 Jeonmin-Dong, Yuseong-gu, Daejeon 305-811, Republic of Korea

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ABSTRACT

Homologous-recombination (HR)-dependent repair defective cells are hypersensitive to poly (ADP-ribose) polymerase (PARP) inhibitors. Combinations of defective HR pathway and PARP inhibitors have been an effective chemotherapeutic modality. We previously showed that knockdown of the WD40-repeat containing protein, Uaf1, causes an HR repair defect in mouse embryo fibroblast cells and therefore, increases sensitivity to PARP inhibitor, ABT-888. Similarly, here, we show that ferulic acid reduces HR repair, inhibits RAD 51 foci formation, and accumulates γ-H2AX in breast cancer cells. Moreover, ferulic acid, when combined with ABT-888, renders breast cancer cells become hypersensitive to ABT-888. Our study indicates that ferulic acid in combination with ABT-888 treatment may serve as an effective combination chemotherapeutic agent as a natural bioactive compound.

1. Introduction

Inhibition of DNA damage repair pathway is a common mechanism by which conventional cancer therapies kill cancer cells. Chemical inhibitors of poly (ADP-ribose) polymerase (PARP) are efficient in inducing sensitivity to BRCA-deficient tumors through synthetic lethality by targeting base-excision repair (BER) in HR-deficient tumors [3,6]. Several suppression factors or defective genes in the HR pathway become sensitive to PARP inhibitors, such as ABT-888 (Veliparib), in tumors [1,16]. Previously, we have reported that a defective Usp1-associated factor 1 (Uaf1) regulates HR repair genes such as Rad 51 in mouse embryo fibroblast cells (MEFs), and makes them hypersensitive to PARP inhibitors [12]. The Uaf1 knockout MEFs increases the genomic instability due to impaired HR pathway. Moreover, epigenetic HR pathway suppression by microRNAs (miRNAs) shows more substantial respond to PARP inhibitors [15]. We previously showed that these miRNAs such as miRNA-1255b, miRNA-148b*, and miRNA193b* are involved in post-transcriptional regulation of HR genes, BRCA1, BRCA2, and,RAD 51, therefore, causing breast and ovarian cancer cells sensitive to PARP inhibitors in a cell-based screening [4].

Ferulic acid is a potent phenolic antioxidant [5]; [10]. Ferulic acid has been shown its therapeutic effects against cancer [9], diabetes [11], cardiovascular disease [13], and neurodegerative disorders [18]. The combination of ferulic acid and α-tocotrienol has been presented as a strategy for cancer therapy (Eitsuka et al., 2014). The combination of ferulic acid and α-tocotrienol markedly reduced breast cancer cell proliferation relative to ferulic acid or α-tocotrienol alone. More cancer cells were accumulated in G1 phase by p21 up-regulation with the combination of ferulic acid and α-tocotrienol.

Despite of various studies demonstrating efficiency of combination therapy through down-regulation of HR pathway and PARP inhibition, the effects of HR suppression by chemotherapeutic agents from natural bioactive compounds are far less understood.

In the current study, we demonstrated that ferulic acid results in impediment to HR repair, inhibition of RAD 51 foci formation assembly, and accumulation of γ-H2AX protein levels in breast cancer cells. Furthermore, we examined that ferulic acid sensitizes HR-proficient breast cancer cell to PARP inhibitor, ABT-888, suggesting that the impacts are mediated through inhibition of HR-dependent pathway.

* Corresponding author. Fax: +82 42 629 8789.
E-mail address: eunmi_park@hnu.kr (E. Park).

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2. Material and methods

2.1. Cell culture

MDA-MB-231, MCF-7 and U2OS cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). The cells were cultured with either ferulic acid (Sigma) or ABT-888 (Selleck) treatment for experiments. Ferulic acid was dissolved in DMSO and ABT-888 was dissolved in PBS for the experiments. All of cell lines were incubated at 37 °C with 5% CO_2.

2.2. Homologous recombination (HR) assay

HR activity was analyzed by DR-GFP$^+$ reporter assay as previously described [12,14,17]. In briefly, U2OS-DR-GFP$^+$ cells carrying a chromosomally integrated single copy of HR repair substrate were used. Double strand break (DSB)-induced HR after I-SceI expression in these cells results in expression of GFP. DR-GFP-positive cells were transfected with an I-SceI plasmid. 48–96 h after induction of chromosomal DSBs through the expression of I-SceI DR-GFP-positive cells were subjected to FACS analysis to quantify the percentage of GFP-positive cells. The siRNA against human BRCA1 were as described [4].

2.3. Immunofluorescence

The RAD 51 and γ-H2AX foci formation were detected by immunofluorescence as described [12] using anti-RAD 51 (Santa cruz) or γ-H2AX (Bethyl lab) primary antibodies and Alex Flour 488 or 568- conjugated secondary antibodies. In brief, MDA-MB-231 cells were pre-extracted with 0.5% Triton/PBS and fixed

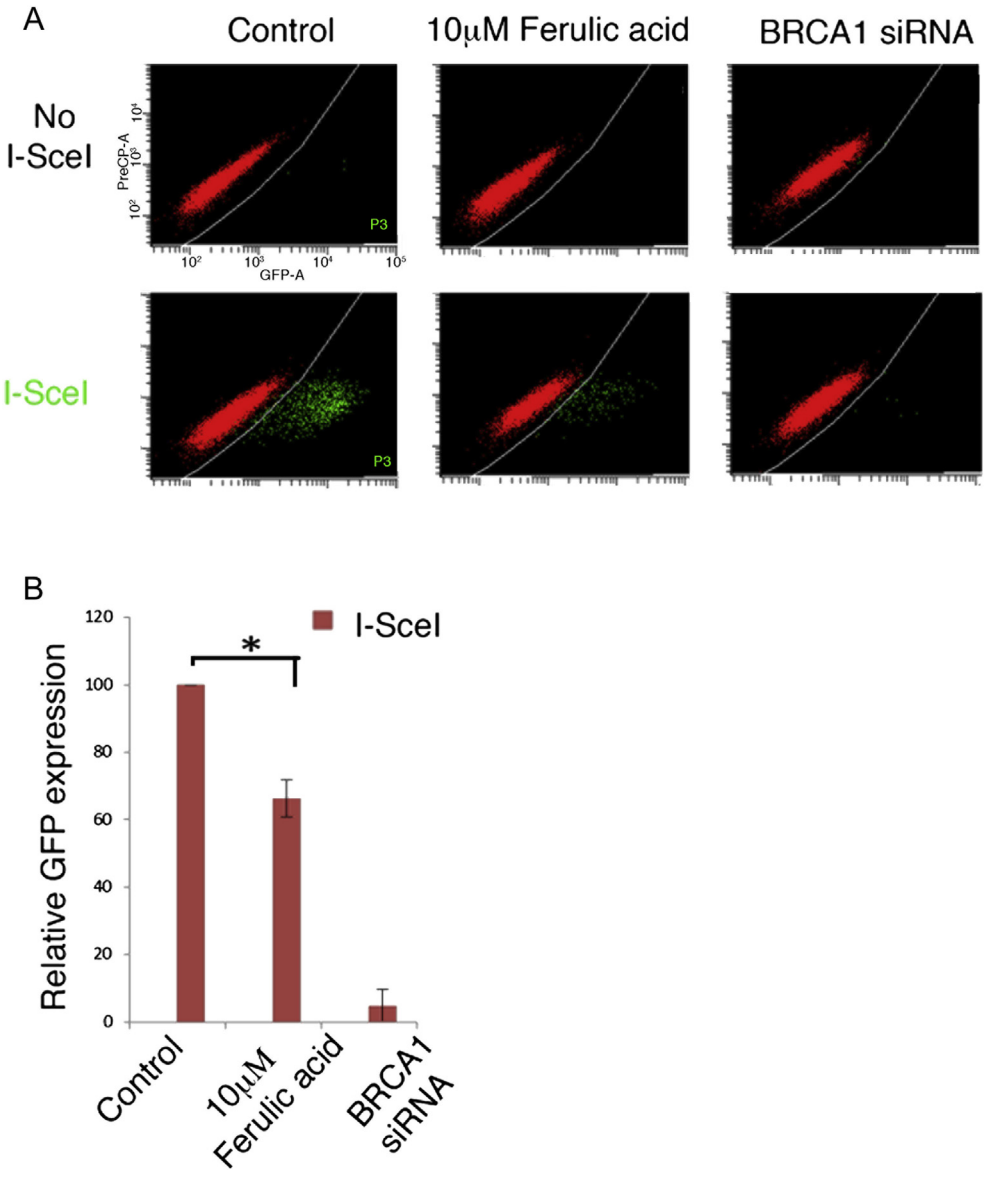

Fig. 1. Ferulic acid inhibits Homologus-recombination (HR)-dependent repair. (A–B) DR-GFP-positive U2OS cells were cultured and prepared with Ferulic acid (10 μM) or DMSO for 24 h. U2OS cells were transfected with siRNA against BRCA1 as a positive control, a homologous recombination protein. DR-GFP U2OS cells were transfected with the I-SceI plasmid for 48 h. GFP positive cells were determined using flow cytometry (FACS). Representative images are shown in (A). The GFP-positive expression as a readout of HR activity is shown. The data are from mean ± standard errors from three independent experiments. (B) GFP-positive cells (Green, P3) were analyzed by flow cytometry (FACS). *; $p < 0.05$. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

with 4% formaldehyde, followed by the staining with the antibodies. The quantification of cells with foci was performed by counting the number of cells with foci using Nikon Zenesis microscope and images were acquired using Axion vision 4.8.2. software.

2.4. Colony assay (cell survival analysis)

MDA-MB-231 and MCF-7 breast cancer cells were prepared for colony assay [12]. After the pre-treatments with ferulic acid or DMSO, the cells were plated in plates. The cells were cultured with

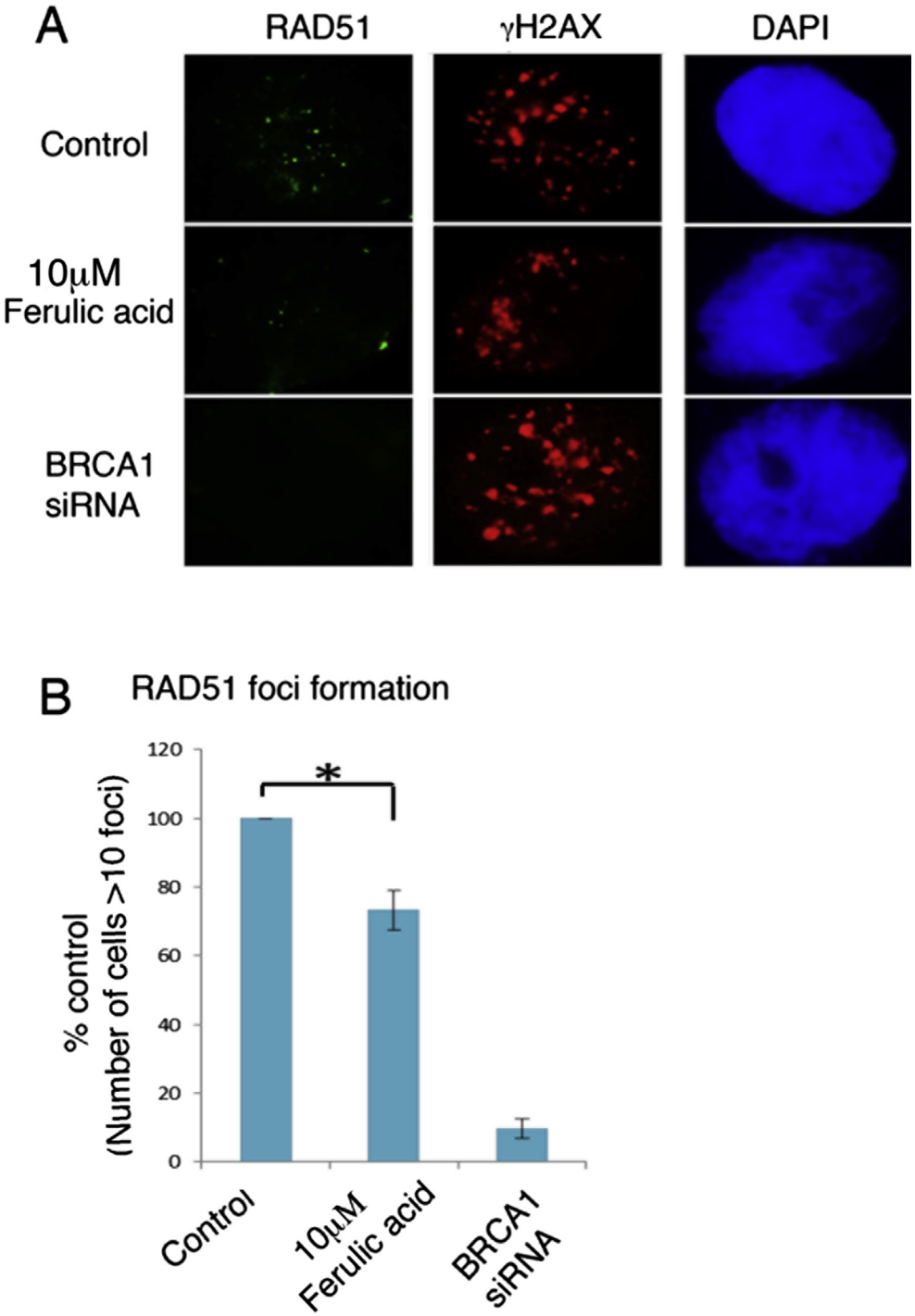

Fig. 2. Fefulic acid inhibits homologous-recombination repair protein, RAD 51 foci formation. (A–B) Analysis of HR repair by RAD 51 foci formation. MDA-MB-231 cells were cultured in 6-well plates and pretreated with ferulic acid (10 μM) or DMSO (negative control) for 24 h. Cells were transfected with siRNA of BRCA1 as a positive control. Cells were stained with RAD51 (Green), γ-H2AX (Red) and DAPI (Blue) for 6 h after exposure to IR 10 Gy. RAD 51 foci positive cells (with>10 foci/a cell) were quantified by comparing 100 cells; the images were shown in (A) and mean ± standard errors from three independent experiments were shown in (B). *; $p < 0.05$. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

ABT-888 for clonogenic assay in triplicates. After 2 weeks in culture, colonies were fixed with methanol and stained with crystal violet.

2.5. Western blot

Cell lysates were prepared as described [12]. In briefly, 20–30 μg of cell lysates were resolved in 10% SDS-PAGE and then transferred to nitrocellulose membranes. Equal loading and transfer of proteins was verified by Ponceau red staining of the membranes and by analyzing H2AX expression. Western blotting was performed using following antibodies: anti-γ H2AX (Cell signaling) and anti- H2AX (Bethyl laboratory).

2.6. Statistical analysis

All data are representative of at least three independent experiments. Data are mean ± SEM unless otherwise indicated. Statistical significance of comparison between two groups was determined by two-tailed Student's *t*-test where indicated. For comparing more than one group, one-way ANOVA was used. Significant differences were considered at *p*-values of less than 0.05.

3. Results

3.1. Ferulic acid reduces homologous-recombination-dependent repair

First, we determined whether ferulic acid impacted an HR directly using DR-GFP + reporter assay (Park et al., 2013b; [14,17]. By using U2OS cells stably expressing DR-GFP + reporter system in which non-functional GFP is repaired to functional GFP if a DSB, by restriction at I-SceI, is repaired by HR. We tested the change in GFP-positive population with ferulic acid.

Ferulic acid treatment decreased percentages of DR-GFP positive cells compared to cells treated with DMSO as a negative control. Substantially decreased percentages of DR-GFP-positive cells were observed in cells infected with siRNA against BRCA1, which is required for HR (See Fig. 1A and B).

As shown in Fig. 1B, FACS profiles of DR-GFP^{+} HR assay show that ferulic acid causes significant reduction in number of GFP-positive cells as shown in green color scatter plots (Fig. 1A) compared to the cells treated with DMSO post to the I-SceI plasmid transduction. Those cells transfected with siRNA against BRCA1, as well as in a positive control, dramatically decreased GFP-positive expressing cells.

3.2. Ferulic acid reduces RAD 51 foci formation for HR-dependent DNA repair

To assess the possibility of ferulic acid as a combination therapy with PARP inhibitor, we examined the effect of ferulic acid on HR repair machinery after introducing a double strand DNA break (DSB). RAD 51 is required in HR pathway and it is widely accepted to test RAD 51 foci formation after DNA damage as a measurement of HR efficiency [2,6]. We pretreated BRCA1 proficient breast cancer cell line, MDA-MB-231 [8], with ferulic acid and measured RAD51 foci formation after IR. siRNA against BRCA1 was used as a positive control for HR inhibition. As expected, siRNA against BRCA1 showed inefficient formation of RAD 51 foci in MDA-MB-231 cells. Interestingly, there was about 25% reduction in RAD 51 foci formation in breast cancer cells treated with 10 μM ferulic acid in response to IR (See Fig. 2A and B). This suggests that ferulic acid inhibits RAD 51 foci formation at DNA damage site.

3.3. Ferulic acid accumulates DNA damage response

Furthermore, we determined whether ferulic acid could induce accumulation of DNA damage. We performed a western blot to examine increased γ-H2AX levels in MDA-MB-231 breast cancer cells followed by ferulic acid treatment. As expected, ferulic acid alone increased γ-H2AX protein levels (Fig. 3; lane 1 verse lane 5). Taken together, ferulic acid increased DNA damage response and inhibited HR repair in breast cancer cell line.

3.4. Ferulic acid sensitizes breast cancer cells to PARP inhibitor

We next examined whether ferulic acid sensitizes breast cancer cell line treated with PARP inhibitor by performing colony formation assays. Quantification of colonies of breast cancer cells, that formed post to the treatment of curcumin and/or PARP inhibitor, was performed to measure drug sensitivity. MDA-MB-231 cells with ferulic acid were more sensitive to PARP inhibitor compared to the cells with DMSO (See Fig. 4A). To assess a possible cell specificity, we tested another HR proficient breast cancer cell line, MCF-7, to ABT-888. As in MDA-MB-231 cells, ferulic acid resulted in increased sensitivity to PARP inhibitor in MCF-7 cells (Fig. 4B).

4. Discussion

Recent studies indicate that HR repair deficient cancer cells are sensitive to PARP inhibitor, ABT-888 [3,6]. We previously characterized that mice deficient with deubiquitinating enzyme subunit of the complex such as Uaf1 die at embryonic day 7.5 and are developmentally retarded [12]. Moreover, Uaf1$^{-/-}$ cells have chromosome instability, a hypersensitivity to ABT-888, and a defect in homologous-recombination repair. Recent study also showed that reduced cdk1 activity impairs BRCA1 function as well as HR repair in lung tumor cells [8]. The combination treatment of cdk1 inhibitor and PARP inhibitors induces tumor regression in the mouse tumor models.

A cell-based screening study of over 1600 compounds reported that bortezomib, curcumin as a bioactive food compound, and MG132 inhibit FANCD2 foci formation, a gene of HR repair post to IR in human cancer cell lines [7]. Moreover, the combination of ferulic acid as the bioactive compound and α-tocotrienol has been reported for chemotherapy.

Here, we hypothesized that ferulic acid impedes HR-dependent repair in breast cancer cells and therefore, ferulic acid in combination with PARP inhibitor, make cancer cells more hypersensitive. In the present study, we demonstrate ferulic acid reduces HR repair, inhibits RAD 51 foci formation, which is a crucial protein in HR repair and reduces HR-dependent repair. Moreover ferulic acid results in accumulation of γ-H2AX, which is a hallmark of DNA damage.

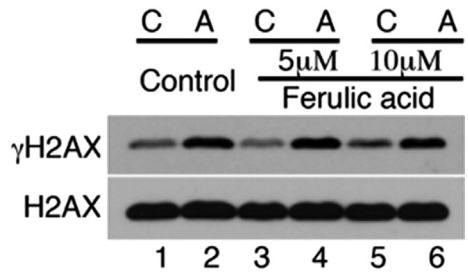

Fig. 3. Ferulic acid accumulates γH2AX levels. Western blotting of lysates in MDA-MB-232 cells with ferulic acid treatment for 24 h. Anti-γH2AX and H2AX antibody were used for immunoblots. C indicates DMSO for a negative control, A indicates ABT-888 treatment (100 μM) for 24 h *; $p < 0.05$.

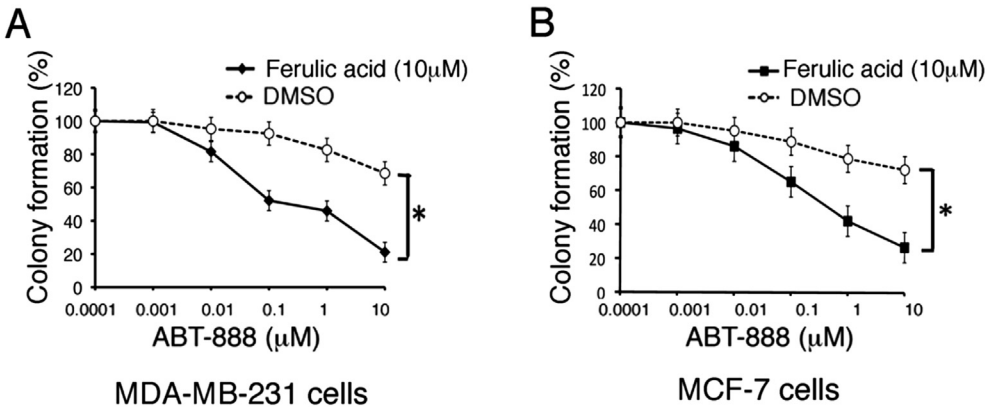

Fig. 4. Breast cancer cells with ferulic acid treatment are highly sensitive to PARP inhibitor. MDA-MB-231 cells (A) and MCF-7 cells (B) were pretreated with ferulic acid (10 μM) or DMSO for 24 h and re-plated in culture dishes. Then the cells were exposed with ABT-888 (0.0001–10 μM) for one week. Survival was determined using a colony assay from three independent experiments. The data are mean ± standard errors. *; $p < 0.05$.

Finally, we demonstrated that combined treatment of ferulic acid and PARP inhibitor reduces colony formation in HR-proficient-breast cancer cells. Ferulic acid causes breast cancer cells become more hypersensitive when co-treated with PARP inhibitor, as compared to the single treatment, which suggests that ferulic acid in combination with PARP inhibitor could be a potential chemo-therapeutic strategy based on synthetic lethality.

Taken together, our study provides new data that ferulic acid regulates HR-dependent-repair, including RAD 51 foci formation, and it hypersensitizes breast cancer cells to PARP inhibitor when combined as a chemotherapy reagent.

Conflict of interest

None.

Acknowledgments

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.147.

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