Biochemical and Biophysical Research Communications 431 (2013) 146–151

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Fulvestrant radiosensitizes human estrogen receptor-positive breast cancer cells

Jing Wang [a,b], Qifeng Yang [a,*], Bruce G. Haffty [c], Xiaoyan Li [b], Meena S. Moran [d,*]

[a] Department of Breast Surgery, Qilu Hospital, Shandong Univeristy, Wenhua Xi Road 107, Shandong Province, PR China
[b] Department of Oncology, Affiliated Hospital of Qingdao University Medical College, Shandong Province, PR China
[c] Department of Radiation Oncology, UMDNJ-Robert Wood Johnson School of Medicine, Cancer Institute of New Jersey, NB, USA
[d] Department of Therapeutic Radiology, Yale University School of Medicine, New Haven, CT, USA

ARTICLE INFO

Article history:
Received 18 December 2012
Available online 10 January 2013

Keywords:
Fulvestrant
Breast conservation therapy
Radiation
Hormonal therapy
Sequencing

ABSTRACT

The optimal sequencing for hormonal therapy and radiation are yet to be determined. We utilized fulvestrant, which is showing promise as an alternative to other agents in the clinical setting of hormonal therapy, to assess the cellular effects of concomitant anti-estrogen therapy (fulvestrant) with radiation (F + RT). This study was conducted to assess the effects of fulvestrant alone vs. F + RT on hormone-receptor positive breast cancer to determine if any positive or negative combined effects exist. The effects of F + RT on human breast cancer cells were assessed using MCF-7 clonogenic and tetrazolium salt colorimetric (MTT) assays. The assays were irradiated with a dose of 0, 2, 4, 6 Gy ± fulvestrant. The effects of F + RT vs. single adjuvant treatment alone on cell-cycle distribution were assessed using flow cytometry; relative expression of repair proteins (Ku70, Ku80, DNA-PKcs, Rad51) was assessed using Western Blot analysis. Cell growth for radiation alone vs. F + RT was 0.885 ± 0.013 vs. 0.622 ± 0.029 @2 Gy, 0.599 ± 0.045 vs. 0.475 ± 0.054 @4 Gy, and 0.472 ± 0.021 vs. 0.380 ± 0.018 @6 Gy RT ($p = 0.003$). While irradiation alone induced G2/M cell cycle arrest, the combination of F + RT induced cell redistribution in the G1 phase and produced a significant decrease in the proportion of cells in G2 phase arrest and in the S phase in breast cancer cells ($p < 0.01$). Furthermore, levels of repair proteins DNA-PKcs and Rad51 were significantly decreased in the cells treated with F + RT compared with irradiation alone. F + RT leads to a decrease in the surviving fraction, increased cell cycle arrest, down regulating of nonhomologous repair protein DNA-PKcs and homologous recombination repair protein RAD51. Thus, our findings suggest that F + RT increases breast cancer cell radiosensitivity compared with radiation alone. These findings have salient implications for designing clinical trials using fulvestrant and radiation therapy.

1. Introduction

Annually, the incidence of breast cancer is over 1,000,000 across the globe with 400,000 deaths [1], making it the most common malignancy of women worldwide. With early detection and improved therapy options, breast cancer mortality appears to be declining during the past few decades [2–4]. Treatment strategies include local/regional management in addition to systemic treatment with cytotoxic chemotherapy, endocrine therapy, biologic therapy or combinations of these. The optimal sequencing of these treatments remains an area of evolution.

Current clinical practice guidelines recommend radiation be delivered after adjuvant chemotherapy in the setting of breast-conservation [5,6], but the optimal sequencing with adjuvant hormonal therapy has only been partially determined. Two clinical studies suggest a detrimental interaction of concomitant tamoxifen and chemotherapy [7,8], with sequential treatment having significantly improved disease-free survival and a trend towards improved survival compared to concomitant treatment. The optimal sequencing of radiotherapy with endocrine therapy remains to be determined. While an antagonistic effect of tamoxifen (TAM) and radiation (RT) has been reported in several in vitro studies, these findings have not been confirmed by laboratory or clinical data [9–11]. In this study, we performed in vitro effects of Fulvestrant (ICI 182,780), the newest generation of endocrine therapy which blocks estrogen at the receptor level and inhibits estrogen-stimulated cell division. Fulvestrant has been found to be as effective as the third-generation aromatase inhibitors (AIs) anastrozole and letrozole for advanced, post-menopausal, tamoxifen-resistant breast cancer, and also appears to be effective after treatment with non-steroidal AIs [12]. To date, there are no published data regarding the interaction of

* Corresponding authors. Addresses: Department of Breast Surgery, Qilu Hospital, Shandong University, Ji'nan, Shandong 250012, PR China. Fax: +86 053182169268 (Q. Yang), Department of Therapeutic Radiology, Yale University School of Medicine, PO Box 208040 New Haven, CT 203-785-6384, USA. Fax: +1 203 785 4622 (M.S. Moran).

E-mail addresses: wangstella5@163.com (J. Wang), qifengy@gmail.com (Q. Yang), hafftybg@umdnj.edu (B.G. Haffty), xiaoyanli1219@gmail.com (X. Li), meena.moran@yale.edu (M.S. Moran).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.01.006

fulvestrant with RT. In the present in vitro study, the direct effects of fulvestrant on radiated breast cancer cells and explored the mechanisms of interaction.

2. Materials and methods

2.1. Cell culture

MCF-7 human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Rockville, IN, USA) with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (FBS, Haoyang Biological Manufacture Co. Ltd., Tianjin, China). The cells were grown as monolayers at 37 °C in a humidified 5% CO_2 incubator with a phenol red-free DMEM medium using 5% fetal bovine serum (HyClone, defined grade, Logan, UT), which supported proliferation of MCF7 cells with an efficacy similar to 30–60 p mol/L E2 [13,14]. Cells were harvested using 0.5 g/l trypsin (Gibco Laboratories) and 0.2 g/l EDTA (Gib-co Laboratories) for 3 min.

2.2. Reagents

Fulvestrant (Sigma Chemical Co., St. Louis, MO) was dissolved in 100% dimethyl sulfoxide (DMSO) to make stock concentrations. HRP-labeled secondary antibodies (KPL, Gaithersburg, MD, USA), antibodies against Ku70, Ku86, DNA-PKcs and Rad51 (Santa Cruz Biotechnology, CA, USA), MTT (3-[4,5-dimethyl-2-yl]-2,5-diphenyl tetrazoliu- m bromide) and anti-β-actin mouse monoclonal antibody (Sigma Chemical Co., St. Louis, MO) were utilized.

2.3. Cell proliferation assay

Cells were plated in 96-well culture plates at a concentration of 2000 cells/well (growth inhibition assay) for 24 h, then treated with 0, 10, 100, 1000 nM concentrations of fulvestrant at 37 °C, 5% CO_2 for 2, 4, 6 days, respectively, with 20 μl MTT in each well. Cells were incubated for another 4 h and 100 μl/well dimethylsulfoxide (DMSO, Sangon Biotech, Shanghai, China) was added to dissolve the formazan after removing the culture medium. The plates were read at 490 nM using Microplate Reader (Bio-Rad, Hercules, CA, USA). The culture medium was used for blank controls and cells without reagents as negative controls.

2.4. Ionizing radiation treatment

MCF-7 cells were irradiated using a Faxitron Cabinet X-ray System (Faxitron X-ray Corp., Wheeling, IL, USA) at room temperature. The X-rays were filtered through a 0.5 mM aluminum filter resulting in a dose rate of 0.4 Gy/min.

2.5. Effect of F + RT by clonogenic assay

The survival and proliferation potential of cells treated with fulvestrant and/or ionizing radiation were assessed by clonogenic assays. Initially, cells were treated with the vehicle control (DMSO) or 100 nM fulvestrant for 4 days and then irradiated (0, 2, 4, or 6 Gy) at room temperature. Cells were trypsinized, suspended in complete medium, counted and re-plated in 60-mM tissue culture dishes allowing formation of macroscopic colonies. Serial dilutions of irradiated cells were plated within 15 min after irradiation. Plates were incubated at 37 °C for 14–21 days, fixed methanol-fixed, Giemsa-stained, and colonies containing >50 cells were counted. The surviving fraction after each X-ray dose (SF_{dose}) was calculated based on the survival of non-irradiated cells treated with the vehicle or fulvestrant, using Survival (S) after a radiation dose (D) fit by a weighted, stratified, linear regression according to the linear–quadratic formula $S(D) = S(0)e^{(-\alpha D-\beta D2)}$, where α and β describe survival curve characteristics that classify cellular response to radiation. To calculate α and β, an SPSS data file was created for each radiation dose–survival curve separately (i.e. ±pretreatment) [15].

2.6. Effect of F + RT by MTT assay

Cells treated with vehicle control (DMSO) or 100 nM fulvestrant for 4 days were irradiated at given doses, then trypsinized, suspended in complete medium, and seeded into 96-well microtitration plates (200 μl per well) at a concentration of 2500–3000 cells/well. Cells were incubated for 6 days (37 °C, 5% CO_2 and 20 μl MTT) and then 4 h (DMSO 100 μl/well) to dissolve the formazan after removing the culture medium. The plates were read at a wavelength of 490 nM. Controls were as above.

2.7. Cell cycle analysis by flow cytometry

After treatment, cells were harvested with trypsin, washed with PBS and then stained with buffer including 50 g/ml propidium iodide (Sigma, St. Louis, MO, USA), for 30 min at room temperature. For fluorescence-activated cell sorting (FACS) analysis, data were collected using a FACSCalibur (BD Bioscience, San Jose, CA, USA) flow cytometer and analyzed by ModFit (Verty, Topsham, ME). The cell-cycle distribution was evaluated by counting >20,000 cells/sample.

2.8. Western blot analysis

The primary antibodies that were utilized including Ku70, Ku80, DNA-PKcs, Rad51 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA USA) and β-actin (1:5000, Sigma–Aldrich, St. Louis, MO, USA) were used. Cells were washed twice with cold phosphate-buffered saline (PBS) and lysed on ice in RIPA buffer (1× PBS, 1% NP40, 0.1% sodium dodecyl sulfate (SDS), 5 mM EDTA, 0.5% sodium deoxycholate and 1 mM sodium orthovanadate) with protease inhibitors and quantified by BCA method [16]. Nuclear and cytosolic extracts were prepared as per manufacturer's protocol. Equal amount of protein (50–80 μg) were separated by SDS polyacrylamide gel, electrotransferred to polyvinylidene fluoride membranes (ImmobilonP; Millipore, Bedford, MA) and blocked in 5% nonfat dry milk in Tris–bufferedsaline, pH 7.5 (100 mM NaCl, 50 mM Tris, 0.1% Tween-20). Membranes were immunoblotted overnight at 4 °C with anti-ku70 monoclonal antibody, anti-ku86 monoclonal antibody, anti-rad51 monoclonal antibody, anti DNA-PKcs monoclonal antibody anti-beta- catenin and anti Bcl-2 monoclonal antibody, followed by their respective horseradish peroxidase conjugated secondary antibodies. Signals were detected by enhanced chemiluminescence. β-actin was used as the endogenous control.

2.9. Statistical analysis

Each portion of the experiment was repeated independently three times. The data are presented as means ± SD of the mean and analyzed with SPSS 17.0 and Microsoft Excel software. Differences between individual groups were analyzed by paired *t*-test and *p* values of <0.05 were considered statistically significant.

3. Results

3.1. Effects of fulvestrant on cellular proliferatio

As shown in Fig. 1, in pre-treated breast cancer cells post-incubation, fulvestrant exposure had a cytotoxic effect on breast cancer cells in a time- and dose-dependent manner. The IC_{50} (concentration of fulvestrant inhibiting cell growth by 50%) were 824.1 ± 2.9 nM, 213.8 ± 2.3 nM, 12.9 ± 1.1 nM on 2, 4, 6 days, respectively. After 4 days, the relative survival fraction of 100 nM fulvestrant was 58.9 ± 1.8%.

3.2. Effects of fulvestrant on breast cancer cells receiving ionizing radiation

Based on the above results, 100 nM fulvestrant and 4 days was used as the optimal pretreatment concentration/time. Cells were

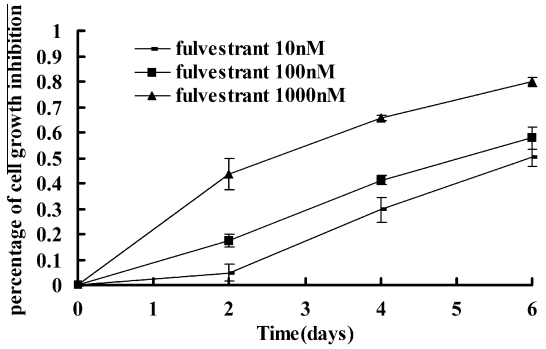

Fig. 1. The effect of fulvestrant on the growth of MCF-7 cells treated with 0, 10, 100, 1000 nM for 0, 2, 4, 6 days. Results shown as a percentage of treated compared with untreated cells.

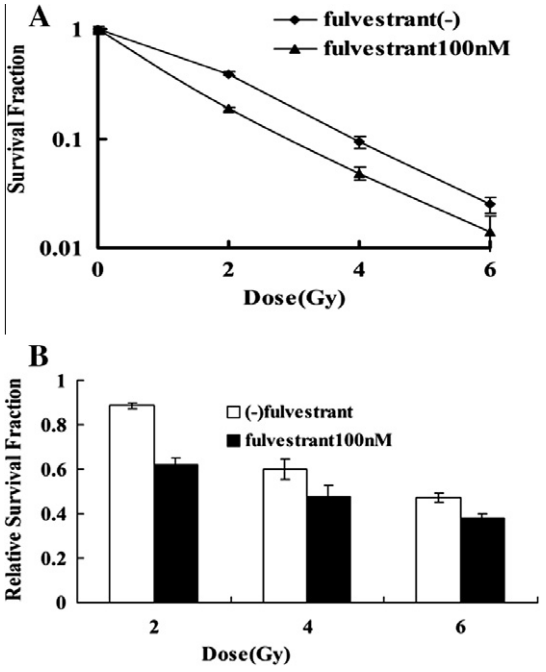

Fig. 2. (A) Survival curves of cancer cells after radiation by clonogenic survival assay treated with fulvestrant (100 nM) or vehicle control (DMSO) for 4 days. Survival shown as the relative plating efficiencies of cells radiated compared to control. Data points are averages of two independent experiments each plated in triplicate ($P < 0.01$). (B) Effect of fulvestrant (100 nM) on radiosensitivity after exposure to 0, 2, 4, 6 Gy compared with vehicle control (DMSO) for 4 days before irradiation ($P = 0.003$).

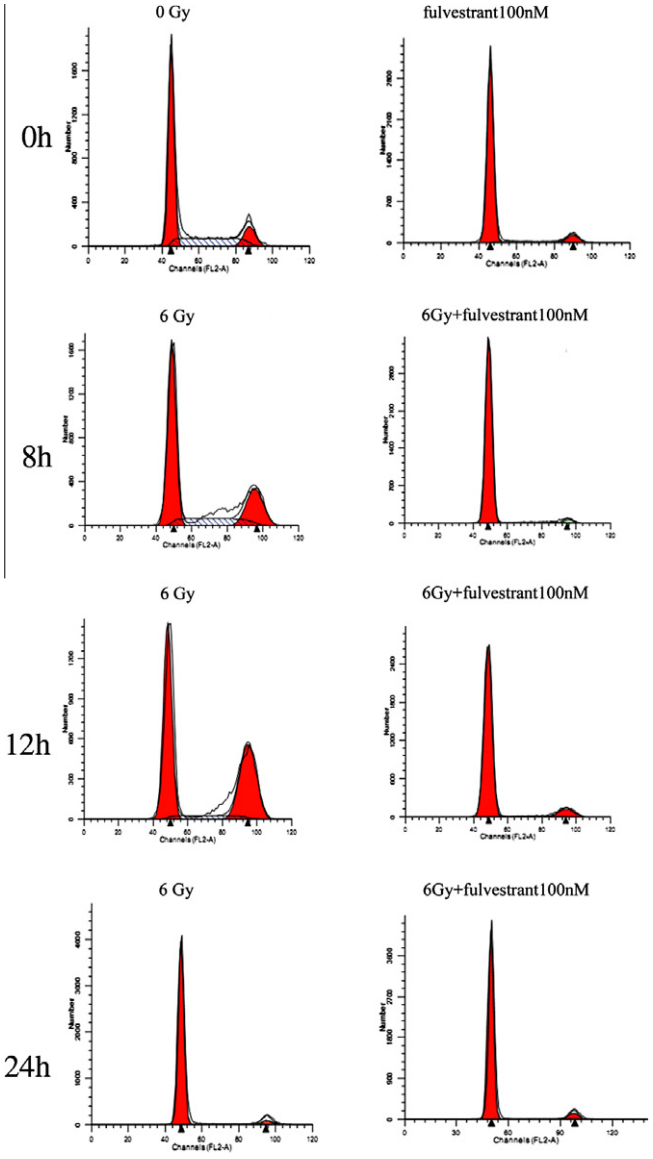

Fig. 3. Flow cytometric analysis of cell cycle distribution: Radiation alone or F + RT (6 Gy) were harvested for 0, 8, 12, 24 h for DNA flow cytometric analysis. Peaks corresponding to G1/G0, the S phase and G2/M phases of the cell cycle are shown.

irradiated with increasing fractions, as described above; these results are shown in Fig. 2A. The SF2 was 0.390 ± 0.013 with irradiation alone and reduced to 0.186 ± 0.007 with fulvestrant pretreatment, reducing the combined treatment SF2 by nearly 30% ($p = 0.001$). Using the linear quadratic model, the α and β components were 0.50 ± 0.011/Gy and 0.353 ± 0.044/Gy^2 without fulvestrant, and −0.132 ± 0.144/Gy and 1.637 ± 0.604/Gy^2 for the F + RT, with significantly different survival curves ($P < 0.01$). Compared with radiation alone, F + RT was inhibited from 0.885 ± 0.013 to 0.622 ± 0.029 at 2 Gy, from 0.599 ± 0.045 to 0.475 ± 0.054 at 4 Gy, and from 0.472 ± 0.021 to 0.380 ± 0.018 6 Gy ($p = 0.003$) (Fig. 2B), suggesting a possible synergic effect on the growth inhibition of human breast cancer cells with F + RT.

3.3. Cell cycle effects of fulvestrant

Using flow cytometry analysis, pre-treated cells (100 nM fulvestrant, 4 days) induced a higher proportion of cells in the G1 phase (86.3 ± 0.1% vs. 61.9 ± 0.5) and a decreased proportion of cells in the S phase (6.5 ± 0.2% vs. 27.7 ± 1.6) compared with controls

Table 1
Cell cycle distribution in MCF-7 cell line after treatment with fulvestrant (100 nM) and irradiation (%).

	0 h		8 h		12 h		24 h	
	Control	Ful	6 Gy	Ful + 6 Gy	6 Gy	Ful + 6 Gy	6 Gy	Ful + 6 Gy
G1	61.9 ± 0.5	86.3 ± 0.1*	57.4 ± 2.3	94.1 ± 0.1*	53.5 ± 2.3	89.5 ± 2.8*	83.8 ± 10.8	84.5 ± 9.0
S	27.7 ± 1.6	6.5 ± 0.2*	17.5 ± 0.2	5.0 ± 0.3*	8.7 ± 1.7	3.1 ± 0.5*	3.2 ± 1.3	2.7 ± 0.9
G2/M	10.3 ± 1.9	7.2 ± 0.1	25.1 ± 2.1	0.5 ± 0.4*	37.8 ± 4.0	7.4 ± 2.3*	13.1 ± 12.1	13.3 ± 10.1

Mean ± S.D. (n = 3).
* P < 0.01, unpaired t-test: comparison with control (0 h), comparison with irradiation alone group (8, 12, 24 h).

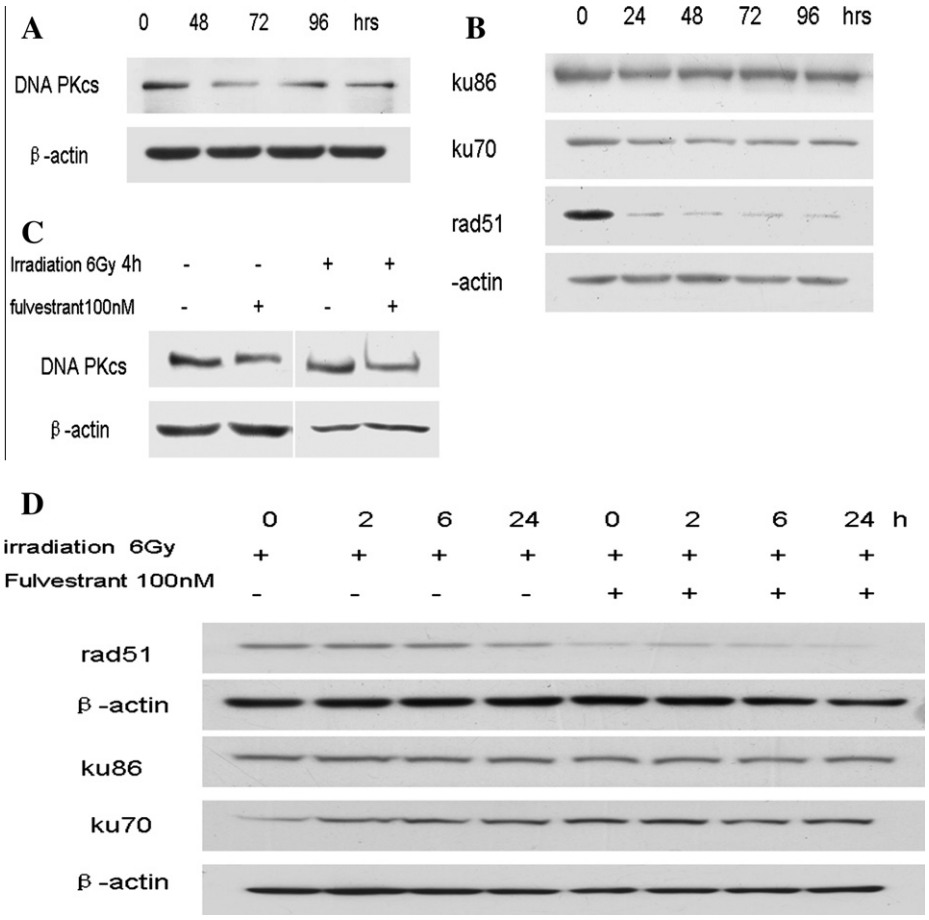

Fig. 4. Western blot analysis for the RAD51, Ku70, Ku86 and DNA-PKcs proteins. (A, B) Cells treated with fulvestrant(100 nM, 24–96 h) had decreased RAD51 and DNA-PKcs protein. (C) Cells treated with fulvestrant(100 nM, 4 days)+6 Gy or radiation alone, harvested (0, 4 h); the protein levels of DNA-PKcs proteins detected. (D) Cells treated (as prior) and protein levels of RAD51, Ku70 and Ku86 detected for 0, 2, 6, 24 h, immunoblotting conducted with anti-RAD51, Ku70, Ku86 and DNA-PKcs antibodies: No changes in Ku70/Ku86, decreased levels of RAD51/DNA-PKcs. Control: Beta-Actin protein.

(Fig. 3 and Table 1). With 6 Gy alone for 8–24 h, a significant cell-cycle arrest in the G2/M phase was observed, with a decrease in the percentage of cells in the G0/G1 phase and the S phase as compared with control. However, F + RT resulted in decreased G2/M phase arrest compared with irradiation alone, with a higher percentage of cells in the G0/G1 phase and a very few of cells in S phase.

3.4. The effects of fulvestrant on proteins DNA-PKcs and RAD51

Using Western blotting, there were no obvious changes in the levels of Ku70 and Ku86 proteins, but RAD51 and DNA-PKcs protein levels were significantly decreased in cells treated with fulvestrant (100 nM/24–96 h), as shown in Fig. 4. In pre-treated cells (100 nM/4 days) before 6 Gy, levels of Ku70 and Ku86 again had no observed change, but the levels of DNA-PKcs and RAD51 protein decreased significantly. However, these changes were not demonstrated in cells treated with irradiation alone.

4. Discussion

Historically, the interactions of endocrine therapy and irradiation in breast cancer have been conflicting. The first in vitro investigation of tamoxifen and irradiation on breast cancer cells was published by Wazer et al. [17], who reported that MCF-7 breast carcinoma cells treated with tamoxifen and radiation were less radiosensitive, resulting in a widened shoulder on survival curves compared with controls. In contrast, when proliferation of breast cancer cultures was inhibited with 17B-estradiol, increased radiosensitivity with a diminished survival curve shoulder was demon-

strated. While these experimental observations were also confirmed by others [18], conflicting data suggest no modulation of radiosensitivity in tamoxifen MCF-7 cells [19]. More importantly, both retrospective and randomized clinical studies do not support the laboratory-observed antagonistic interaction of XRT and tamoxifen [10,11,20–22]. These conflicting results may be a result of characteristics of different breast cancer cell lines, or theoretically, in vitro medium conditions (i.e. in vitro experiments using phenol-red or fetal bovine serum may mimic estrogenic activity, affecting these results, which may not be reproduced in clinical studies).

Third-generation aromatase inhibitors (AI), which have been shown to improve disease-free survival in a variety of adjuvant settings for early breast cancer [23,24], are an alternative to tamoxifen for post-menopausal breast cancer. However, limited data on the use of AIs with radiotherapy exist. Azria et al. reported the effects of RT and letrozole results in sensitization in breast cancer cells in aromatase-expressing breast tumor cells(MCF-7CA) transfected with the CYP19 gene in vitro compared with radiation alone [25]. In a phase 2 randomized trial by the same investigators, the effects of concurrent vs. sequential radiotherapy and letrozole were assessed [26], and preliminary results suggest no interaction or effects of concomitant vs. sequential use.

Fulvestrant, a pure estrogen-receptor antagonist that directly competes with estrogen by binding, blocking and increasing degradation of ER protein and subsequent ER signaling, is an effective and well-tolerated drug for treatment of estrogen-sensitive breast cancer [27]. Clinical investigations of Fulvestrant as single or combination therapies are currently underway. In this study, human breast-cancer cells were treated with fulvestrant alone, radiation alone, or in combination to determine their effects on cell survival. We found that the combination of F + RT reduced the SF_2 (fraction of breast cancer cells surviving 2 Gy) by nearly 30%, suggesting that the addition of fulvestrant promotes inhibition of cellular proliferation and enhances the effects of radiation.

In this study, cell cycle effects of fulvestrant increased the proportion of cells in G1 arrest, accompanied by a simultaneous decrease in cells in the S phase. Given that cancer cells are most radiosensitive in the G2/M phase, less sensitive in G0/G1, and least sensitive in the latter part of the S phase [28], and cell cycle progression can be stopped at the G1, S, and G2 checkpoints [29], we postulate that the decreased proportion of cells in S phase before irradiation may result in the observed decrease in the surviving fraction with F + RT. Furthermore, the increased proportion of cells arrested in the G2 phase in F + RT treatment group may also contribute to the decrease of the surviving fraction.

In mammalian cells, there are two major pathways to repair potentially lethal DNA double-strand breaks (DSBs): homologous recombination (HR) and nonhomologous DNA end joining (NHEJ), with NHEJ as the predominant repair process for cells in G0, G1 or early S-phase, with mutant cell lines lacking key components of this pathway all exhibiting impaired kinetics of DNA DSB repair and exquisite radiosensitivity [30]. Homologous recombination (HR) is a more precise repair mechanism and is more important for the repair of DSB in late-S and G2 when a sister chromatid is available for the recombination reaction [31]. RAD51 is a central player in the HR-mediated repair of DSB. DNA-PKcs, along with Ku70/Ku80, Artemis nuclease, and the XRCC4/DNS-Ligase IV complex, function to mediate nonhomologous DNA end joining [32]. Based on our findings of decreased levels of DNA-PKcs and RAD 51 proteins with fulvestrant administration, we hypothesize that the radiosensitizing effect of fulvestrant on breast cancer cells may involve impairment of DNA double-strand breaks (DSBs) repair. Furthermore, in F + RT cells, the levels DNA-PKcs and RAD 51 were also significantly decreased compared with RT alone, providing yet another possible explanation for the observed increase in radiosensitivity in the combined treatment arm.

In summary, our findings suggest that the concomitant use of F + RT sensitizes human breast cancer cells to the ionizing effects of radiation by increasing cell cycle arrest and down-regulating proteins (DNA-PKcs and RAD51) involved in double-strand DNA break repair in comparison to breast cancer cells treated with radiation alone. These findings suggest that F + RT has a synergic effect in arresting cellular proliferation in human breast cancer cells. Our findings are the first to report this radiosensitization effect of fulvestrant, which may have important implications for breast cancer translational research and will need further investigation in both the laboratory and clinical settings.

Acknowledgments

This project is supported by National Natural Science Foundation of China (Nos. 81072150; 81172529; 81272903) and also partially funded by the Kalimeris Fund, Department of Therapeutic Radiology, Yale University School of Medicine.

References

[1] H. Igene, Global health inequalities and breast cancer: an impending public health problem for developing countries, Breast J. 14 (2008) 428–434.
[2] A. Jemal, R. Siegel, J. Xu, E. Ward, Cancer statistics, 2010, CA cancer, J. Clin. 60 (2010) 277–300.
[3] Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials, Lancet 365 (2005) 1687–1717.
[4] A. Jemal, M.M. Center, C. DeSantis, E.M. Ward, Global patterns of cancer incidence and mortality rates and trends, Cancer Epidemiol. Biomarkers Prev. 19 (2010) 1893–1907.
[5] J.R. Bellon, S.E. Come, R.S. Gelman, I.C. Henderson, L.N. Shulman, B.J. Silver, J.R. Harris, A. Recht, Sequencing of chemotherapy and radiation therapy in early-stage breast cancer: updated results of a prospective randomized trial, J. Clin. Oncol. 23 (2005) 1934–1940.
[6] A. Recht, S.E. Come, I.C. Henderson, R.S. Gelman, B. Silver, D.F. Hayes, L.N. Shulman, J.R. Harris, The sequencing of chemotherapy and radiation therapy after conservative surgery for early-stage breast cancer, N. Engl. J. Med. 334 (1996) 1356–1361.
[7] K.S. Albain, W.E. Barlow, P.M. Ravdin, W.B. Farrar, G.V. Burton, S.J. Ketchel, C.D. Cobau, E.G. Levine, J.N. Ingle, K.I. Pritchard, A.S. Lichter, D.J. Schneider, M.D. Abeloff, I.C. Henderson, H.B. Muss, S.J. Green, D. Lew, R.B. Livingston, S. Martino, C.K. Osborne, Adjuvant chemotherapy and timing of tamoxifen in postmenopausal patients with endocrine-responsive, node-positive breast cancer: a phase 3, open-label, randomised controlled trial, Lancet 374 (2009) 2055–2063.
[8] C. Pico, M. Martin, C. Jara, A. Barnadas, A. Pelegri, A. Balil, C. Camps, A. Frau, A. Rodriguez-Lescure, J.M. Lopez-Vega, J. De La Haba, A. Tres, I. Alvarez, E. Alba, A. Arcusa, A. Oltra, N. Batista, T. Checa, R. Perez-Carrion, J. Curto, Epirubicin-cyclophosphamide adjuvant chemotherapy plus tamoxifen administered concurrently versus sequentially: randomized phase III trial in postmenopausal node-positive breast cancer patients. A GEICAM 9401 study, Ann. Oncol. 15 (2004) 79–87.
[9] H. Schmidberger, R.M. Hermann, C.F. Hess, G. Emons, Interactions between radiation and endocrine therapy in breast cancer, Endocr. Relat. Cancer. 10 (2003) 375–388.
[10] L.J. Pierce, L.F. Hutchins, S.R. Green, D.L. Lew, J.R. Gralow, R.B. Livingston, C.K. Osborne, K.S. Albain, Sequencing of tamoxifen and radiotherapy after breast-conserving surgery in early-stage breast cancer, J. Clin. Oncol. 23 (2005) 24–29.
[11] P.H. Ahn, H.T. Vu, D. Lannin, E. Obedian, M.P. DiGiovanna, B. Burtness, B.G. Haffty, Sequence of radiotherapy with tamoxifen in conservatively managed breast cancer does not affect local relapse rates, J. Clin. Oncol. 23 (2005) 17–23.
[12] J. Lynn, Fulvestrant ('Faslodex') – a new hormonal treatment for advanced breast cancer, Eur. J. Oncol. Nurs. 8 (Suppl. 2) (2004) S83–S88.
[13] K.R. Coser, J. Chesnes, J. Hur, S. Ray, K.J. Isselbacher, T. Shioda, Global analysis of ligand sensitivity of estrogen inducible and suppressible genes in MCF7/BUS breast cancer cells by DNA microarray, Proc. Natl. Acad. Sci. USA 100 (2003) 13994–13999.
[14] J. Hur, D.W. Bell, K.L. Dean, K.R. Coser, P.C. Hilario, R.A. Okimoto, E.M. Tobey, S.L. Smith, K.J. Isselbacher, T. Shioda, Regulation of expression of BIK proapoptotic protein in human breast cancer cells: p53-dependent induction of BIK mRNA by fulvestrant and proteasomal degradation of BIK protein, Cancer Res. 66 (2006) 10153–10161.
[15] N.A.P. Franken, H.M. Rodermond, J. Stap, J. Haveman, C. van Bree, Clonogenic assay of cells in vitro, Nature Protocols 1 (2006) 2315–2319.

[16] X. Li, X. Kong, Q. Huo, H. Guo, S. Yan, C. Yuan, M.S. Moran, C. Shao, Q. Yang, Metadherin enhances the invasiveness of breast cancer cells by inducing epithelial to mesenchymal transition, Cancer Sci. 102 (2011) 1151–1157.
[17] D.E. Wazer, O.F. Tercilla, P.S. Lin, R. Schmidt-Ullrich, Modulation in the radiosensitivity of MCF-7 human breast carcinoma cells by 17B-estradiol and tamoxifen, Br. J. Radiol. 62 (1989) 1079–1083.
[18] M. Villalobos, D. Becerra, M.I. Nunez, M.T. Valenzuela, E. Siles, N. Olea, V. Pedraza, J.M. Ruiz de Almodovar, Radiosensitivity of human breast cancer cell lines of different hormonal responsiveness. Modulatory effects of oestradiol, Int. J. Radiat. Biol. 70 (1996) 161–169.
[19] J.N. Sarkaria, E.M. Miller, C.J. Parker, V.C. Jordan, R.T. Mulcahy, 4-Hydroxytamoxifen, an active metabolite of tamoxifen, does not alter the radiation sensitivity of MCF-7 breast carcinoma cells irradiated in vitro, Breast Cancer Res. Treat. 30 (1994) 159–165.
[20] B. Fisher, J. Dignam, J. Bryant, A. DeCillis, D.L. Wickerham, N. Wolmark, J. Costantino, C. Redmond, E.R. Fisher, D.M. Bowman, L. Deschenes, N.V. Dimitrov, R.G. Margolese, A. Robidoux, H. Shibata, J. Terz, A.H. Paterson, M.I. Feldman, W. Farrar, J. Evans, H.L. Lickley, Five versus more than five years of tamoxifen therapy for breast cancer patients with negative lymph nodes and estrogen receptor-positive tumors, J. Natl. Cancer Inst. 88 (1996) 1529–1542.
[21] B. Fisher, J. Dignam, N. Wolmark, D.L. Wickerham, E.R. Fisher, E. Mamounas, R. Smith, M. Begovic, N.V. Dimitrov, R.G. Margolese, C.G. Kardinal, M.T. Kavanah, L. Fehrenbacher, R.H. Oishi, Tamoxifen in treatment of intraductal breast cancer: national surgical adjuvant breast and bowel project B-24 randomised controlled trial, Lancet 353 (1999) 1993–2000.
[22] K. Dalberg, H. Johansson, U. Johansson, L.E. Rutqvist, A randomized trial of long term adjuvant tamoxifen plus postoperative radiation therapy versus radiation therapy alone for patients with early stage breast carcinoma treated with breast-conserving surgery. Stockholm Breast Cancer Study Group, Cancer 82 (1998) 2204–2211.
[23] B. Thurlimann, A. Keshaviah, A.S. Coates, H. Mouridsen, L. Mauriac, J.F. Forbes, R. Paridaens, M. Castiglione-Gertsch, R.D. Gelber, M. Rabaglio, I. Smith, A. Wardley, K.N. Price, A. Goldhirsch, A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer, N. Engl. J. Med. 353 (2005) 2747–2757.
[24] F. Boccardo, A. Rubagotti, M. Puntoni, P. Guglielmini, D. Amoroso, A. Fini, G. Paladini, M. Mesiti, D. Romeo, M. Rinaldini, S. Scali, M. Porpiglia, C. Benedetto, N. Restuccia, F. Buzzi, R. Franchi, B. Massidda, V. Distante, D. Amadori, P. Sismondi, Switching to anastrozole versus continued tamoxifen treatment of early breast cancer: preliminary results of the Italian Tamoxifen Anastrozole Trial, J. Clin. Oncol. 23 (2005) 5138–5147.
[25] D. Azria, C. Larbouret, S. Cunat, M. Ozsahin, S. Gourgou, P. Martineau, D.B. Evans, G. Romieu, P. Pujol, A. Pelegrin, Letrozole sensitizes breast cancer cells to ionizing radiation, Breast Cancer Res. 7 (2005) R156–163.
[26] D. Azria, Y. Belkacemi, G. Romieu, S. Gourgou, M. Gutowski, K. Zaman, C.L. Moscardo, C. Lemanski, M. Coelho, B. Rosenstein, P. Fenoglietto, N.E. Crompton, M. Ozsahin, Concurrent or sequential adjuvant letrozole and radiotherapy after conservative surgery for early-stage breast cancer (CO-HO-RT): a phase 2 randomised trial, Lancet Oncol. 11 (2010) 258–265.
[27] C.K. Osborne, E.B. Coronado-Heinsohn, S.G. Hilsenbeck, B.L. McCue, A.E. Wakeling, R.A. McClelland, D.L. Manning, R.I. Nicholson, Comparison of the effects of a pure steroidal antiestrogen with those of tamoxifen in a model of human breast cancer, J. Natl. Cancer Inst. 87 (1995) 746–750.
[28] T.M. Pawlik, K. Keyomarsi, Role of cell cycle in mediating sensitivity to radiotherapy, Int. J. Radiat. Oncol. Biol. Phys. 59 (2004) 928–942.
[29] M.B. Kastan, Cell cycle. Checking two steps, Nature 410 (2001) 766–767.
[30] Z. Mao, Y. Jiang, X. Liu, A. Seluanov, V. Gorbunova, DNA repair by homologous recombination, but not by nonhomologous end joining, is elevated in breast cancer cells, Neoplasia 11 (2009) 683–691.
[31] A. Choudhury, A. Cuddihy, R.G. Bristow, Radiation and new molecular agents part I: targeting ATM-ATR checkpoints, DNA repair, and the proteasome, Semin. Radiat. Oncol. 16 (2006) 51–58.
[32] M.R. Lieber, The mechanism of human nonhomologous DNA end joining, J. Biol. Chem. 283 (2008) 1–5.