Aromatase Inhibitor–Mediated Downregulation of INrf2 (Keap1) Leads to Increased Nrf2 and Resistance in Breast Cancer

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Abstract

Aromatase inhibitors are effective drugs that reduce or eliminate hormone-sensitive breast cancer. However, despite their efficacy, resistance to these drugs can occur in some patients. The INrf2 (Keap1):Nrf2 complex serves as a sensor of drug/radiation-induced oxidative/electrophilic stress. INrf2 constitutively suppresses Nrf2 by functioning as an adapter protein for the Cul3/Rbx1-mediated ubiquitination/degradation of Nrf2. Upon stress, Nrf2 dissociates from INrf2, is stabilized, translocates to the nucleus, and coordinately induces a battery of cytoprotective gene expression. Current studies investigated the role of Nrf2 in aromatase inhibitor resistance. RT-PCR and immunoblot assays showed that aromatase inhibitor–resistant breast cancer LTLTCa and AnaR cells express lower INrf2 and higher Nrf2 protein levels, as compared with drug-sensitive MCF-7Ca and AC1 cells, respectively. The increase in Nrf2 was due to lower ubiquitination/degradation of Nrf2 in aromatase inhibitor–resistant cells. Higher Nrf2-mediated levels of biotransformation enzymes, drug transporters, and antiapoptotic proteins contributed to reduced efficacy of drugs and aversion to apoptosis that led to drug resistance. shRNA inhibition of Nrf2 in LTLTCa (LTLTCa-Nrf2KD) cells reduced resistance and sensitized cells to aromatase inhibitor exemestane. Interestingly, LTLTCa-Nrf2KD cells also showed reduced levels of aldehyde dehydrogenase, a marker of tumor-initiating cells and significantly decreased mammosphere formation, as compared with LTLTCa-Vector control cells. The results together suggest that persistent aromatase inhibitor treatment downregulated INrf2 leading to higher expression of Nrf2 and Nrf2-regulated cytoprotective proteins that resulted in increased aromatase inhibitor drug resistance. These findings provide a rationale for the development of Nrf2 inhibitors to overcome resistance and increase efficacy of aromatase inhibitors. Mol Cancer Ther; 14(7): 1728–37. ©2015 AACR.

Introduction

Drug resistance is the major obstacle to the successful treatment of many cancers (1). The factors that contribute to the development of drug resistance include alterations in drug intake, efflux, metabolism, and excretion. Deregelation of cell death by evasion of apoptosis, necrosis, mitotic catastrophe, or senescence also contributes to drug resistance (2–5). In addition, the differential expression of membrane proteins such as solute carriers, channels, and ATP-binding cassette transporters have all been demonstrated to play important roles in drug resistance (4, 5).

Breast cancer is the most common cancer among women (6). Aromatase inhibitors are an effective first line of treatment for ERα-positive breast cancer that constitutes three-fourth of all types of breast cancers (7). Aromatase (cytochrome P450 CYP19A1) catalyzes the rate-limiting and essential step of estrogen biosynthesis; the aromatization of androgens to estrogens (8, 9). Breast cancer tissues have been shown to express aromatase and produce higher levels of estrogens than noncancerous cells (7). Estrogens stimulate breast cancer cell growth and proliferation. Aromatase inhibitors became the choice of treatment for breast cancer in postmenopausal women because they block the synthesis of estrogens required by cancer cells to grow (10). Currently, there are three aromatase inhibitors approved by the FDA, letrozole, anastrozole, and exemestane (Supplementary Fig. S1). These are approved for postmenopausal women with hormone receptor–positive breast cancer in both the adjuvant and metastatic setting. Letrozole is more potent than other aromatase inhibitors in reducing plasma estrogen levels (11). While aromatase inhibitors are a very effective treatment, their benefit is often limited by the emergence of resistance that occurs in a significant number of patients in the adjuvant setting and is inevitable in the metastatic setting.

The Inrf2 (Keap1):Nrf2 complex acts as a cellular sensor of xenobiotics, drugs, and radiation-induced ROS/electrophilic stress (12). Nuclear factor Nrf2 controls the expression and coordinated induction of a battery of genes encoding detoxifying enzymes [quinone oxidoreductases (NQO1 and NQO2), glutathione S-transferases, heme oxygenase 1 (HO-1)], glutathione and related proteins [glutathione, thioredoxins, γ-glutamyl cysteinyl synthetase (γ-GCS)], ubiquitination enzymes and proteasomes (12, 13), drug transporters (MRP; refs. 14, 15), and antiapoptotic proteins (16). Nrf2 is retained in the cytoplasm by an inhibitor INrf2 or Keap1 (17, 18). INrf2 functions as an adapter...
for Cul3/Rbx1-mediated degradation of Nrf2 (12). In response to chemical/drug/radiation including antioxidant tert-butyl hydroquinone (t-BHQ)-induced oxidative/electrophilic stress, Nrf2 is switched on (separation from INrf2 and stabilization of Nrf2) and then off (ubiquitination and degradation of Nrf2) by distinct early and delayed mechanisms (12). Oxidative/electrophilic modifications of Nrf2 cysteine151 and/or PKC phosphorylation of Nrf2 serine40 result in the escape or release of Nrf2 from INrf2 (12). Nrf2 is stabilized and translocates to the nucleus, forms heterodimers with small Maf or Jun proteins, and binds antioxidant response elements resulting in coordinated activation of gene expression (12). Indeed, in vivo evidence has demonstrated the importance of Nrf2 in protecting cells from the toxic and carcinogenic effects of many environmental insults. Nrf2-knockout mice were susceptible to acute damages induced by acetaminophen, ovalbumin, cigarette smoke, and pentachlorophenol and mice were susceptible to acute damages induced by acetaminophen, ovalbumin, cigarette smoke, and pentachlorophenol and had increased tumor formation when exposed to carcinogens such as benzo[a]pyrene, diesel exhaust, and N-nitrosobutyl (4-hydroxybutyl) amine (19–22). Therefore, Nrf2 appears to play a significant role in cytoprotection and cell survival (12). In addition, Nrf2 plays significant role in prevention of cancer metastasis (23–25).

Studies have also described the detrimental effects of Nrf2 (26–30). Persistent stabilization and nuclear accumulation of Nrf2 is suggested to play a role in survival of cancer cells and drug resistance. Increase in Nrf2 due to inactivating mutations in INrf2 has been reported in lung cancer (26, 27). Although Nrf2 is thought to contribute to drug resistance by inducing cytoprotective proteins (28, 29), its role in resistance of breast cancer to aromatase inhibitors remains unknown.

The studies in this report showed that aromatase inhibitor-resistant breast cancer cells contain lower INrf2 and higher Nrf2 levels, as compared with drug-sensitive cells. Studies also revealed that higher Nrf2 was due to decreased INrf2, lower ubiquitination, and slower degradation of Nrf2 in aromatase inhibitor-resistant cells. Higher Nrf2-mediated increase in bio-transformation enzymes, drug transporters, and antiapoptotic proteins contributed to reduced efficacy of drugs and prevention of apoptosis that led to drug resistance. Interestingly, LTLTCa cells deficient in Nrf2 (LTLTCa-Nrf2KD) showed reduced levels of aldehyde dehydrogenase (ALDH), a marker of tumor-initiating cells (TIC), significantly decreased mammosphere formation and increased sensitivity to exemestane and doxorubicin, as compared with parental LTLTCa-Vector control (LTLTCa-V), respectively.

Materials and Methods

Chemicals and reagents

Puromycin dihydrochloride (sc-108071), control shRNA lentiviral particles-A (sc-108080), Nrf2 shRNA (sc-37030-V), anti-Nrf2 (sc-13032), anti-Kerp1 (sc-15246), anti-HO-1 (sc-10789), anti-NQO1 (sc-32793), anti-Bcl-2 (sc-492), anti-Bcl-xL (sc-8392), anti-Mcl-1 (sc-819), anti-Lamin B (sc-6217), anti-Mdr-1 (sc-8318), anti-MRP1 (sc-13960), anti-HER2 (sc-284), anti-Ub (sc-8017), anti-Ku70 (sc-1778), antibodies were from Santa Cruz Biotechnology. Glutathione assay kit (item No. 703002) was from Cayman Chemical. Ultra-low attachment of 24-well plate (cat. no. 3473) for mammospheres was obtained from Corning. DCFDA Cellular ROS Detection Assay Kit (cat. no. ab113851) and γ-glutamylcysteine synthetase (GGCL, ab40929) antibody were obtained from Abcam. Anti-LDH (cat. no. 3558) from Cell Signaling Technology, anti-MRP4 (cat. no. ALX-801-038) from Enzo Life Sciences, anti-BCRP (cat. no. OP191-200HIL), Ku80 (cat. no. NA54), and proteasome inhibitor MG-132 (cat. no. 474790) from Millipore were purchased for Western blotting. Aldehyde assay kit was obtained from Stem Cell Technologies. Aromatase inhibitors (letrozole and anastrozole) were provided by Dr. Brodie’s laboratory.

Cells and cell culture conditions

Aromatase inhibitor-sensitive cells (MCF-7Ca and AC1) and aromatase inhibitor-resistant cells (LTLTCa and AnaR) have been described previously (31–33). Briefly, human breast cancer MCF-7 cells were stably transfected with the human aromatase gene to generate MCF-7Ca cells (32). Letrozole-resistant LTLTCa cells were isolated from MCF-7Ca mouse xenograft tumors treated with letrozole for 56 weeks. The lower expression of ERβ in aromatase inhibitor-resistant cells (LTLTCa and AnaR) as compared with aromatase inhibitor-sensitive cells was previously described (34). However, we did observe as is already published (31) that LTLTCa cells express significantly less ERα than MCF-7Ca cells. Similar to MCF-7Ca and LTLTCa cells, AC1 cells were generated from the MCF-7 cells by stable transfection with human aromatase gene. AnaR cells were aromatase-resistant cells isolated from AC1 mouse xenograft tumors treated with anastrozole for 14 weeks (31). MCF-7Ca cells were grown in DMEM containing 100 μg/ml G418 sulfate and 5% FBS. DMEM with 700 μg/ml G418 sulfate and 10% FBS were used to culture AC1 cells. LTLTCa cells were maintained in phenol red–free modified Improved Minimum Essential Medium (IMEM) containing 700 μg/ml G418 sulfate, 1 μmol/L letrozole and 5% charcoal-stripped FBS. AnaR cells were grown in modified IMEM with 700 μg/ml G418 sulfate, 20 μmol/L anastrozole, and 10% charcoal-stripped FBS. The LTLTCa-Nrf2 knock down (LTLTCa-Nrf2KD) cells were cultured in modified IMEM medium supplemented with 700 μg/ml G418 sulfate and 5% charcoal-stripped FBS. MCF-7Ca, LTLTCa, AC1, and AnaR cells were grown in monolayer in medium containing 1% penicillin/streptomycin in an incubator at 37°C with 95% air and 5% CO2.

Generation of stable LTLTCa cells expressing Nrf2 shRNA

LTLTCa cells were transduced with Nrf2 shRNA or control shRNA lentiviral particles and cells stably expressing Nrf2 shRNA or control shRNA were selected in the presence of 10 μg/ml puromycin and designated as LTLTCa-Nrf2 knockdown (LTLTCa-Nrf2KD) and LTLTCa-Vector control (LTLTCa-V), respectively.

Western blotting

Cells were untreated or treated with proteasome inhibitors MG-132 or epoxomicin or DMSO vehicle control. The cells were washed with cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 0.2 mmol/L EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate) supplemented with 1× protease inhibitor (Roche Applied Science). Subcellular fractionation of the cells was performed according to manufacturer’s protocol (Active Motif). Proteins were quantified using Bio-Rad protein assay. The cell lysates

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(30–50 μg) were separated on SDS-PAGE and transferred to nitrocellulose membranes. The membranes after blocking in 5% nonfat milk solution in Tris buffered Saline Tween-20 (TBST) were incubated with the primary antibodies overnight at 4°C and washed four times with TBST. This was followed by incubation with secondary antibody at room temperature for 1 hour and washed four times with TBST. The protein bands were visualized using chemiluminescence (ECL) system (Thermo Scientific, product no. 32209). ImageJ software (NIH, Bethesda, MD) was used to quantify the intensity of protein bands. The protein bands were normalized against loading controls.

Degradation assay
Cells were treated with 25 μg/mL cycloheximide for the indicated time points, washed twice with ice-cold 1× PBS, and lysed in RIPA buffer with protease inhibitors. Thirty micrograms of total cell lysate was loaded per well of 10% SDS-PAGE gel, transferred, and immunoblotted with Nrf2 and β-actin antibodies. Nrf2 band intensity was quantified and normalized to β-actin. The relative levels of Nrf2 from sample with zero (0) minute was considered as initial level. The graphs represent the natural logarithm of the relative levels of the Nrf2 protein as a function of the cycloheximide chase time. The half-life of protein was determined in the linear range of the degradation curve.

Immunoprecipitation and ubiquitination assay
For ubiquitination assay, cells were treated with 2 μM of MG-132 for 16 hours and lysed in RIPA buffer. One milligram of whole-cell lysate was immunoprecipitated with 1 μg of rabbit IgG or Nrf2 antibody by incubating the reaction mixture overnight in RIPA buffer supplemented with 0.1% SDS at 4°C. After adding 20 μL of washed protein A/G plus beads (Santa Cruz Biotechnology), the mixture was incubated for 2 hours at 4°C and centrifuged at 4,000 rpm for 1 minute. The beads were washed twice with RIPA buffer. Thirty micrograms of SDS sample dye was added to each tube and boiled for 5 minutes and immunoprecipitated Nrf2 was separated by 8% SDS-PAGE and immunoblotted with anti-ubiquitin antibody and the same blot was reprobed for Nrf2.

Cell survival assay
MCF-7Ca, LTLTCa, and LTLTCa-Nrf2KD cells were seeded at the density of 10,000, 20,000, and 20,000 cells per well, respectively, in 24-well plates. After 24-hour incubation, cells were treated with different concentrations of exemestane (viz. 0, 5, 10, 20, and 30 μM) for 72 hours. The cells were incubated with freshly prepared MTT dye (200 μL/well of 3 mg/mL MTT dye in PBS) for 2 hours. MTT dye is reduced by mitochondria aldehyde dehydrogenase to form insoluble formazan crystals. The amount of formazan produced is proportional to viable cells. After dissolving formazan crystals in DMSO, absorbance was recorded spectrophotometrically at 570 nm. Cell viability was calculated from absorbance and normalized to the value of the corresponding vehicle control cells. Each data point represents a mean ± SD from three independent experiments.

Aldefluor staining
ALDEFLUOR assay (Stem Cell Technologies) was performed according to the manufacturer’s instructions. MCF-7Ca and LTLTCA cells and LTLTCA-V and LTLTCA-Nrf2KD cells expressing ALDH were stained with Aldefluor reagent and identified by comparing the same sample with and without the ALDH inhibitor diethylaminoenzaldehyde (DEAB). Cells were acquired using FACS Canto and analyzed using Flowjo software (BD Biosciences). Dead cells were excluded on the basis of light scatter characteristics and using viability dye (propidium iodide) gating parameters.

Isolation of TICs using Aldefluor staining
Aldefluor assay/Aldehyde dehydrogenase assay (Stem Cell Technologies) was performed according to the manufacturer’s instructions. Briefly, LTLTCA cells were stained with Aldefluor reagent along with the inhibitor of ALDH, DEAB, and sorted using FACS ARIA (BD Biosciences). All cells showing differential ALDH-staining pattern were sorted and designated as ALDH-high and ALDH-low cells based on highest and lowest expression of ALDH enzyme, respectively.

Mammosphere assay
Mammosphere assay was performed using reagents from Stem Cell Technologies, as per manufacturer’s instructions. Briefly, LTLTCA-V and LTLTCA-Nrf2KD cells were suspended in complete MammoCult medium and 10,000 cells per well were plated in ultra-low attachment 24-well plates. Mammospheres were counted after 3 weeks. Stabilized spheres with a colony count of at least 50 cells were considered as mammospheres (34).

Gene expression analysis
Total RNA was isolated from the untreated cells and cells treated with DMSO or t-BHQ for the indicated time periods using RNeasy mini kit, following manufacturer’s protocol. cDNA was synthesized from 1 μg of total RNA as template and the cDNA was used to determine the target gene expression by quantitative real-time PCR using TaqMan gene expression assays.

ROS detection
DCFDA Cellular ROS detection assay kit was used to measure the cellular levels of ROS. Cells were trypsinized and washed with PBS. The cells were suspended in 2’,7’-dichlorofluorescein diacetate (DCFDA) and incubated at 37°C for 30 minutes in the dark and washed with 1× buffer. A total of 105 DCFDA-stained cells were suspended in 1 mL of 1× supplemented buffer and 105 cells in 100 μL of the cell suspension were added to each well of 96-well black plate. A total of 50 μmol/L of t-butyl hydroperoxide was added and the cells were incubated at 37°C for 3 hours to generate ROS as positive control. Using TECAN Infinite M1000 PRO plate reader, ROS-mediated fluorescence intensity was recorded with excitation wavelength at 485 nm and emission wavelength at 535 nm.

Glutathione quantification
Total glutathione content was determined spectrophotometrically using Cayman’s glutathione assay kit following the manufacturer’s protocol. Briefly, the cells were seeded on 6-well plates on day 1 and harvested on day 3 and lysed in 1× buffer supplied in Glutathione detection kit and oxidized and reduced form of glutathione was quantified following the kit protocol using TECAN plate reader (405 nm). Glutathione content is expressed as μmol/L/μg protein.
Statistical analyses

Data from cell survival, cell death assay, and real-time PCR were analyzed using a two-tailed Student test. Data were presented as the mean ± SD. Two datasets with \( P < 0.05 \) were considered as statistically significant.

Results

Aromatase inhibitor–resistant cells contain higher ROS, lower INrf2, and higher Nrf2 protein levels, as compared with sensitive cells

Letrozole-sensitive MCF-7Ca and -resistant LTLTCa cells were analyzed for ROS and immunoblotted for Nrf2, INrf2, Nrf2-regulated proteins, and actin (Fig. 1). The results demonstrated that drug-resistant LTLTCa cells contain higher ROS, lower INrf2, and higher Nrf2 levels, as compared with drug-sensitive MCF-7Ca cells (Fig. 1A and B). Subcellular fractionation followed by immunoblotting analysis revealed that nuclear Nrf2 was significantly higher in LTLTCa cells, as compared with MCF-7Ca cells (Fig. 1C). In the same experiment, the cytosolic fraction did not show Nrf2 in either LTLTCa or MCF-7Ca cells (Fig. 1C). The resistant LTLTCa cells also demonstrated significantly increased Nrf2-regulated GCLC (catalytic subunit of glutathione synthesizing enzyme γ-GCS), heme oxygenase-1 (HO-1), drug transporters (MRP-1, MRP-4, and BCRP), and antiapoptotic (Bcl-xL and Mcl-1) proteins, as compared with sensitive MCF-7Ca cells (Fig. 1D). Further analysis of letrozole-sensitive and -resistant cells demonstrated an increase in total and reduced glutathione in resistant LTLTCa cells, as compared with sensitive MCF-7Ca cells (Fig. 1E).

In similar experiments, a second cell line AnaR that is resistant to another aromatase inhibitor anastrozole, also showed lower INrf2, higher Nrf2, and GCLC levels, as compared with drug-sensitive AC1 cells (Fig. 1F). In addition, the AnaR cells showed increased expression of Nrf2 downstream genes encoding detoxifying enzymes (GCLC, HO-1), as compared with drug-sensitive AC1 cells (Fig. 1F). Together, these results indicate that persistent treatment of cells with aromatase inhibitors increase ROS, decreases INrf2, increases nuclear Nrf2, increases expression of Nrf2-regulated genes and levels of reduced glutathione, and suggest that Nrf2 and Nrf2-regulated genes play a role in aromatase inhibitor resistance. Interestingly, both letrozole-resistant LTLTCa and anastrozole-resistant AnaR cells containing higher levels of Nrf2 showed downregulation of the Nrf2 downstream gene NQO1, as compared with sensitive cells (Supplementary Fig. S2). The reasons for downregulation of NQO1 gene expression in aromatase inhibitor–resistant cells remain unknown. It is noteworthy that the lack of induction of NQO1 gene in letrozole-treated Hepa 1c1c7 cells was observed earlier (35).

Figure 1.

Aromatase inhibitor–resistant cells generated increased levels of ROS and expressed lower levels of INrf2, higher levels of Nrf2, and Nrf2 downstream gene expression. A, cellular ROS–mediated fluorescence in letrozole-sensitive (MCF-7Ca) and letrozole-resistant (LTLTCa) cells. Cells were incubated with DCFDA and cellular ROS–mediated fluorescence intensity was recorded using microplate reader. B, D, and F, MCF-7Ca and LTLTCa cells were lysed and analyzed by Western blot analysis. C, cytosolic and nuclear fractions from MCF-7Ca and LTLTCa cells were determined spectrophotometrically at 570 nm. F, Western blot analysis of the relative levels of Nrf2, INrf2 (Keap1), and Nrf2 target genes anastrozole-sensitive AC1 and anastrozole-resistant AnaR cells.
Khatri et al.

shRNA inhibition of Nrf2 in LTLTCa cells decreased Nrf2 downstream gene expression and increased sensitivity to exemestane

LTLTCa cells were transduced with either lentiviral vector (control) or Nrf2-shRNA viral particles and positive clones selected in puromycin. MCF-7Ca, LTLTCa-V (vector control), and LTLTCa-Nrf2KD (Nrf2 knockdown) cells were immunoblotted for Nrf2 and INrf2; detoxifying proteins GCLC and NQO1; membrane transporters MRP1, MRP4, and BCRP; and antiapoptotic proteins Mcl-1, Bcl-xL, and actin (Fig. 2A and B). shRNA silencing of Nrf2 significantly reduced the levels of Nrf2, GCLC, NQO1, MRP4, Bcl-xL, and Mcl-1 (Fig 2A and B). Nrf2KD cells also showed downregulation of MRP1 but the change was insignificant. This is presumably due to relatively lower contribution of Nrf2, as compared with other factors including NF-kB and c-Jun that regulate expression of MRP1 in LTLTCa cells (15). Previous studies have suggested the option of using steroidal aromatase inhibitor exemestane to treat HER2-negative, hormonal receptor-positive, postmenopausal metastatic breast cancer patients with resistance to nonsteroidal aromatase inhibitor (reviewed in ref. 36). Therefore, one of the aims of the experiment was to evaluate exemestane sensitivity of aromatase inhibitor-resistant LTLTCa cells. The MCF-7Ca, LTLTCa, and LTLTCa-Nrf2KD cells were compared for exemestane sensitivity (Fig. 2C). Interestingly, the treatment of LTLTCa cells (expressing higher Nrf2 compared with MCF-7Ca cells) with exemestane showed some degree of sensitivity that increased with increasing concentration of exemestane (Fig. 2C). However, MCF-7Ca cells containing lower Nrf2 showed significant sensitivity to 20 and 30 μmol/L exemestane as compared with LTLTCa cells containing higher Nrf2 (Fig. 2C). Intriguingly, shRNA inhibition of Nrf2 significantly sensitized LTLTCa-Nrf2KD cells to exemestane (Fig. 2C). The 20 and 30 μmol/L exemestane concentrations significantly decreased cell survival in LTLTCa-Nrf2KD cells as compared with LTLTCa cells (Fig. 2C). Furthermore, the Nrf2 levels in LTLTCa-Nrf2KD cells were similar to MCF-7Ca cells (Fig. 2A) and their sensitivities to exemestane were not significantly different (Fig. 2C; *P > 0.7758). In other words, knockdown of Nrf2 in LTLTCa-Nrf2KD cells sensitized cells to exemestane to a similar extent as observed with MCF-7Ca cells. Interestingly, LTLTCa-Nrf2KD cells also showed increased sensitivity to genotoxic antitumor drugs doxorubicin and etoposide as compared with LTLTCa cells (Supplementary Fig. S3). Together, these results suggested a role for Nrf2 in aromatase inhibitor drug resistance. It is noteworthy that LTLTCa cells contained significantly lower ERα, as compared with MCF-7Ca cells and shRNA inhibition of Nrf2 in LTLTCa cells had more or less no effect on ERα level in LTLTCa cells (Supplementary Fig. S4), indicating that Nrf2 does not regulate ERα expression in resistant cells.

Letrozole-resistant LTLTCa cells show lower Nrf2 ubiquitination levels and a decreased rate of Nrf2 degradation when compared with sensitive MCF-7Ca cells

LTLTCa cells demonstrated decreased Nrf2 ubiquitination and degradation, as compared with MCF-7Ca cells (Fig. 3A). In related experiments, the rate of degradation of Nrf2 was significantly lower in LTLTCa cells compared with MCF-7Ca cells (Fig. 3B). These results collectively suggested that higher levels of Nrf2 in LTLTCa cells are due to decreased ubiquitination and degradation of Nrf2. Notably, INrf2, which functions as an adaptor protein for Cul3-Rbx1-mediated ubiquitination and degradation of Nrf2, is downregulated in aromatase inhibitor-resistant LTLTCa cells (Figs. 1B and 3C). Therefore, it is reasonable to conclude that lower INrf2 levels were responsible for the reduced ubiquitination and degradation of Nrf2 in LTLTCa cells. We also determined whether downregulation of INrf2 in LTLTCa cells is due to degradation and/or decreased transcript levels of the INrf2 gene, as compared with MCF-7Ca cells (Fig. 3C–E). The treatment of

Figure 2. LTLTCa-Nrf2KD cells were more sensitive to exemestane. A and B, Western blot analysis of Nrf2, INrf2, and Nrf2 downstream proteins in MCF-7Ca, LTLTCa, and LTLTCa-Nrf2KD cells. C, comparative sensitivities of MCF-7Ca, LTLTCa, and LTLTCa-Nrf2KD cells to exemestane. The cells were exposed to 10% ethanol in PBS (vehicle control represented by 0 exemestane) and varying concentrations of exemestane (1, 2.5, 5.0, 10, 20, and 50 μmol/L) for 72 hours and analyzed for cell survival by MTT assay. Cell survival obtained at 1 and 2.5 μmol/L has been excluded from the graph as the results were similar to those obtained from 5 μmol/L. Each data point represents a mean ± SD from three independent experiments.
LTLTCa cells showed decreased ubiquitination and degradation of Nrf2 compared with MCF-7Ca cells. A, 1 mg of total cell lysate from MG-132–treated cells was immunoprecipitated with 1 μg of rabbit IgG or Nrf2 antibody. The immunoprecipitated Nrf2 was immunoblotted for ubiquitin and Nrf2. B, cells were treated with 25 μg/mL cycloheximide (CHX) for the indicated time points and 30 μg of total cells lysate was immunoblotted with Nrf2 and β-actin antibodies. The graphs represent the natural logarithm of the relative levels of the Nrf2 protein versus the cycloheximide chase time and the half-life of Nrf2 was determined using the linear part of the degradation curve. LTLTCa cells showed no difference in rate of Nrf2 protein degradation but demonstrated lower levels of Nrf2 transcripts, as compared with MCF-7Ca cells. Cells treated with MG-132 (C) and with epoxomicin (D) were lysed and immunoblotted. E, total RNA was isolated from the cells and cDNA was synthesized from 1 μg of total RNA and the cDNA was used to quantify the INrf2 gene transcripts at basal level.

Figure 3.

LTLTCa cells showed decreased ubiquitination and degradation of Nrf2 compared with MCF-7Ca cells. A, 1 mg of total cell lysate from MG-132–treated cells was immunoprecipitated with 1 μg of rabbit IgG or Nrf2 antibody. The immunoprecipitated Nrf2 was immunoblotted for ubiquitin and Nrf2. B, cells were treated with 25 μg/mL cycloheximide (CHX) for the indicated time points and 30 μg of total cells lysate was immunoblotted with Nrf2 and β-actin antibodies. The graphs represent the natural logarithm of the relative levels of the Nrf2 protein versus the cycloheximide chase time and the half-life of Nrf2 was determined using the linear part of the degradation curve. LTLTCa cells showed no difference in rate of Nrf2 protein degradation but demonstrated lower levels of INrf2 transcripts, as compared with MCF-7Ca cells. Cells treated with MG-132 (C) and with epoxomicin (D) were lysed and immunoblotted. E, total RNA was isolated from the cells and cDNA was synthesized from 1 μg of total RNA and the cDNA was used to quantify the INrf2 gene transcripts at basal level.

INrf2 gene expression is downregulated in aromatase inhibitor–resistant cells. RT-PCR analysis also showed a marginal increase in Nrf2 gene expression in LTLTCa cells, as compared with sensitive MCF-7Ca cells that might also have contributed to higher Nrf2 in resistant cells (Supplementary Fig. S3).

In TICs from LTLTCa cells, lower INrf2 and higher Nrf2 expression levels lead to expression of Nrf2 targets, GCLC, DNA repair proteins, and HER2

Recent studies have implicated mammary TICs in resistance to chemotherapy and radiation (38, 39). TICs are immature, poorly differentiated, and highly tumorigenic (40–42). TICs have a decreased ability to undergo apoptosis and a higher ability for DNA repair, making them more resistant to cancer therapy, compared with differentiated counterparts (43, 44). We isolated TICs expressing ALDH from LTLTCa cell culture. It has been reported that chemoresistant cancer stem cells have high ALDH activity (45) and ALDH is considered as a marker of normal and malignant human mammary stem cells (46). MCF-7Ca, LTLTCa,
TICs isolated from LTLTCa cells expressed lower levels of INrf2 and higher levels of Nrf2. LTLTCa cells were subjected to Aldefluor staining (Aldefluor Staining Kit, Stem Cell Technologies) and the cells expressing ALDH were further gated into ALDH-high and ALDH-low cells. Cells were sorted as ALDH-high (TIC) and ALDH-low (non-TIC) fractions. MCF-7Ca and LTLTCa cells also received the same treatment as LTLTCa-low ALDH and LTLTCa-high ALDH cells. MCF-7Ca and LTLTCa cells were used as control cells. The cells were lysed and total cell lysate was immunoblotted with indicated antibodies. *, Control live cells were sorted by propidium iodide exclusion.

Figure 4.

LTLTCa-low ALDH, and LTLTCa-high ALDH cells were lysed and immunoblotted for Nrf2, INrf2, GCLC, DNA repair proteins, and HER-2 (Fig. 4). Results revealed that TICs expressed lower levels of Nrf2 and higher levels of Nrf2 and HER-2 (Fig. 4). Intriguingly, TICs with high ALDH levels (stem cells) expressed significantly higher levels of nonhomologous end-joining (NHEJ) DNA repair proteins Ku80 and Ku70, as compared with MCF-7Ca and LTLTCa cells. This observation has high significance as TICs are believed to contribute to drug resistance.

shRNA inhibition of Nrf2 in LTLTCa cells significantly decreases TICs and mammosphere formation

MCF-7Ca and LTLTCa cells were immunoblotted for Nrf2 (Fig. 5A) and in separate experiments were stained to assess the expression of stem cell marker ALDH in the absence and presence of ALDH inhibitor DEAB (Fig. 5B). As expected, the levels of Nrf2 and ALDH were significantly higher in LTLTCa cells compared with MCF-7Ca cells (Fig. 5A and B). This indicated the presence of an increased stem cell-like population in LTLTCa cells containing higher levels of Nrf2, as compared with MCF-7Ca cells with lower Nrf2 protein. In related experiments, LTLTCa-V (vector control), LTLTCa-Nrf2KD clone #2 and clone #1 cells were immunoblotted for Nrf2 and actin (Fig. 5C). The results demonstrated that LTLTCa-V cells showed highest expression of Nrf2, which was followed by clone #2 and clone #1 cells LTLTCa-V and the two clones of LTLTCa-Nrf2KD cells were stained for assessing the expression of stem cell marker ALDH in the absence and presence of ALDH inhibitor DEAB (Fig. 5D). Results demonstrated a direct correlation between Nrf2 expression levels and the magnitude of ALDH expression. LTLTCa-V cells expressing the highest levels of Nrf2 led the highest percentage of cells in a gated region R3 that stained positive for ALDH. Moreover, clone #2, containing lower expression levels of Nrf2 demonstrated significantly decreased ALDH. Notably, shRNA downregulation of Nrf2 in LTLTCa (clone #1) containing lowest level of Nrf2 also showed the least staining for ALDH. These results showed that shRNA inhibition of Nrf2 led to an Nrf2-dependent decrease in ALDH-positive TICs. It is noteworthy that ALDH is an Nrf2 downstream gene and its expression is regulated by Nrf2 (47–49). Therefore, our observation of a relationship between Nrf2 and ALDH is strengthened by previous reports (47–49). In related experiments, LTLTCa and both clones of LTLTCa-Nrf2KD cells were also analyzed for mammosphere formation (Fig. 6). The results (compare Figs. 5C and 6) revealed a direct correlation between Nrf2 and mammosphere formation. shRNA inhibition of Nrf2 in LTLTCa cells led to Nrf2 concentration-dependent decrease in mammosphere formation. Together, the results suggest a direct correlation between Nrf2-ALDH-positive TICs and mammosphere formation with implications in aromatase inhibitor drug resistance that warrant further studies.

Discussion

This is the first report demonstrating a role for Nrf2 in aromatase inhibitor resistance in breast cancer. Letrozole-resistant LTLTCa cells generated significantly higher ROS levels, as compared with letrozole-sensitive MCF-7Ca cells. This increase in ROS was more significant considering that reduced glutathione that scavenges ROS was also increased. We believe that long-term treatment of letrozole could result in continuous generation of ROS, which is known to activate Nrf2. However, Nrf2-mediated antioxidant gene expression might not be sufficient to lower the levels of ROS leading to higher levels of ROS in drug-resistant LTLTCa cells, as compared with drug-sensitive MCF-7Ca cells. LTLTCa cells also showed lower INrf2 and higher expression levels of Nrf2, as compared with MCF-7Ca cells. Similar results were also observed in aromatase-resistant AnaR cells. The increase in ROS and decrease in Innr2 led to decreased ubiquitination and degradation of Nrf2 leading to the stabilization and nuclear translocation of Nrf2. High levels of Nrf2 in the nucleus led to coordinated induction of Nrf2 downstream cytoprotective proteins including detoxifying enzymes, membrane transporters, and antiapoptotic proteins. On the basis of the documented role of Nrf2-activated cytoprotective proteins in drug resistance in other systems (28), our results strongly suggest that Nrf2 plays a role in aromatase inhibitor resistance. This was further supported by studies showing that inhibition of Nrf2-sensitive LTLTCa cells to the aromatase inhibitor exemestane. Our studies also revealed that lower levels of Innr2 in drug-resistant LTLTCa cells is not due to instability of Inrr2 protein as inhibitors of proteasomes failed to increase the level of Inrr2. Furthermore, the lower levels of Inrr2 in LTLTCa cells are also not
Nrf2 and Aromatase Inhibitor Drug Resistance

Figure 5.
Increased Nrf2 in LTLTCa cells is directly associated with higher ALDH. A and B, LTLTCa cells containing higher Nrf2 showed increased ALDH. A, Western blot analysis. MCF-7Ca and LTLTCa cells were immunoblotted for Nrf2 and β-actin. B, ALDH measurement. MCF-7Ca and LTLTCa cells were stained with Aldefluor in absence and presence of ALDH inhibitor DEAB and the percentage of cells expressing ALDH was determined. C and D, Nrf2-knocked down LTLTCa cells expressed lower levels of ALDH. Two clones of LTLTCa cells expressing Nrf2shRNA were selected. A, LTLTCa-V and LTLTCa-Nrf2KD cells were lysed and immunoblotted with Nrf2 to confirm reduced levels of Nrf2. B, all the cells were subjected to Aldefluor staining and the percentage of cells expressing ALDH was determined by comparing the same sample with and without the ALDH inhibitor DEAB. LTLTCa-Nrf2KD cells contained lower percentage of cells expressing ALDH. Data were acquired using FACScanto.

Nrf2 in mammosphere development. Therefore, it is reasonable to conclude that higher Nrf2 levels in TICs contributed to aromatase inhibitor resistance.

Collectively, the results led to the hypothesis (Supplementary Fig. S5) that aromatase inhibitor downregulates INrf2 transcripts that, combined with higher ROS, leads to increased Nrf2 and Nrf2 downstream cytoprotective proteins including detoxifying enzymes, antioxidants, and efflux pumps. In addition, higher Nrf2-mediated increased expression of antiapoptotic proteins that led to reduced apoptosis. Finally, higher Nrf2 increased TIC survival and mammosphere formation. Together, all these Nrf2-dependent processes contributed to aromatase inhibitor drug resistance.

Previous studies have shown increased signaling through HER2 receptors and increased MAPK activity as probable causes of endocrine drug resistance (reviewed in refs. 7 and 52). In addition, breast cancer cells receiving long-term antiestrogen treatment appear to have increased ROS and disruption of reversible redox signaling that involves redox-sensitive factors including protein phosphatases, protein kinases such as ERK, and transcription factors AP-1 and NF-xB that contribute to drug resistance (reviewed in ref. 53). Furthermore, PI3K–Akt–mTOR pathway has also been implicated in endocrine drug resistance (54). The INrf2:Nrf2 system identified in the current report is a novel mechanism of aromatase inhibitor drug resistance but is also related to the abovementioned mechanisms. This is because Nrf2 is a transcription factor that controls redox homeostasis (reviewed in ref. 12). In addition, Nrf2 itself is regulated by PI3K–Akt–mTOR, MAPKs, and redox-sensitive factors including AP1 (reviewed in ref. 12). Further studies are required to explore the exact relationship between these factors and pathways leading to aromatase inhibitor drug resistance and possible therapeutic intervention.
Recent studies have shown some benefit in using steroidal aromatase inhibitor exemestane in treating HER2-negative, hormonal receptor–positive, and postmenopausal metastatic breast cancer patients with resistance to nonsteroidal aromatase inhibitor (36). The studies in this report suggest that it might be possible to increase the efficacy of exemestane in the patients with endocrine drug resistance by inhibiting Nrf2. This assumption is based on observation that shRNA inhibition of Nrf2 in letrozole-resistant cells significantly increased the sensitivity to exemestane.

In conclusion, the current studies present strong evidence for a role of INrf2:Nrf2 in aromatase inhibitor drug resistance in breast cancer. The mechanism(s) involving Nrf2 to combat drug resistance is especially interesting as it is estrogen independent. The studies also suggest that Nrf2 inhibitors from natural sources could be explored for use as adjuvants with aromatase inhibitor drugs to treat aromatase inhibitor–resistant breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: R. Khatri, R. Guha, A.E. Tomkinson, A. Brodie, A.K. Jaiswal

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Shah, R. Guha, A. Brodie

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Khatri, R. Guha, A. Brodie, A.K. Jaiswal

Writing, review, and/or revision of the manuscript: R. Khatri, P. Shah, R. Guha, A. Brodie, A.K. Jaiswal

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Acknowledgments

The authors thank their colleagues at the University of Maryland School of Medicine (Baltimore, MD) for helpful discussions.

Grant Support

This work was supported by NIH grant R01 ES012265 (to A.K. Jaiswal), RO1 ES021483 (to A.K. Jaiswal), RO1 CA62483 (to A. Brodie), RO1 GM047466 (to A.K. Jaiswal), and a grant from V-Foundation and Endow Funds (to A.K. Jaiswal).

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Received August 7, 2014; revised April 1, 2015; accepted May 5, 2015; published OnlineFirst May 14, 2015.

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Retraction: Aromatase Inhibitor-mediated Downregulation of INrf2 (Keap1) Leads to Increased Nrf2 and Resistance in Breast Cancer

Raju Khatri, Preeti Shah, Rupa Guha, Feyruz V. Rassool, Alan E. Tomkinson, Angela Brodie, and Anil K. Jaiswal

The article (1) has been retracted at the request of the editors. Following an institutional review by the University of Maryland (Baltimore, MD), it was determined that data used in some of the figures cannot be supported. Specifically, the University's versions on record of Fig. 1E do not match the final published Fig. 1E. The error bars in the published figure are markedly smaller than the University's versions on record. Further analysis showed that histograms depicting averaged data may have been created from a single datum, some samples may have been disconnected from the analysis, and values for error bars were inappropriately set. University investigators also found that data were excluded from analyses presented in Fig. 3 of the published article. The statistical significance reported in the article is nullified when these data points are included. In addition, it was concluded that a number of duplicated data points found in the data were uncorroborated. The University investigators determined that the published figure does not support significant differences between MCF-7Ca and LTLT-Ca, or the authors' hypothesis.

A copy of this retraction notice was sent to the last known email addresses for all seven authors, none of whom responded.

Reference

Published online November 2, 2018.
doi: 10.1158/1535-7163.MCT-17-0310
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Molecular Cancer Therapeutics

Aromatase Inhibitor–Mediated Downregulation of INrf2 (Keap1) Leads to Increased Nrf2 and Resistance in Breast Cancer

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doi:10.1158/1535-7163.MCT-14-0672

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