AKT-Induced Tamoxifen Resistance Is Overturned by RRM2 Inhibition

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Abstract

Acquired tamoxifen resistance develops in the majority of hormone-responsive breast cancers and frequently involves overexpression of the PI3K/AKT axis. Here, breast cancer cells with elevated endogenous AKT or overexpression of activated AKT exhibited tamoxifen-stimulated cell proliferation and enhanced cell motility. To gain mechanistic insight on AKT-induced endocrine resistance, gene expression profiling was performed to determine the transcripts that are differentially expressed post-tamoxifen therapy under conditions of AKT overexpression. Consistent with the biologic outcome, many of these transcripts function in cell proliferation and cell motility networks and were quantitatively validated in a larger panel of breast cancer cells. Moreover, ribonucleotide reductase M2 (RRM2) was revealed as a key contributor to AKT-induced tamoxifen resistance. Inhibition of RRM2 by RNA interference (RNAi)–mediated approaches significantly reversed the tamoxifen-resistant cell growth, inhibited cell motility, and activated DNA damage and proapoptotic pathways. In addition, treatment of tamoxifen-resistant breast cancer cells with the small molecule RRM inhibitor didox significantly reduced in vitro and in vivo growth. Thus, AKT-expressing breast cancer cells upregulate RRM2 expression, leading to increased DNA repair and protection from tamoxifen-induced apoptosis.

Implications: These findings identify RRM2 as an AKT-regulated gene, which plays a role in tamoxifen resistance and may prove to be a novel target for effective diagnostic and preventative strategies. Mol Cancer Res; 12(3); 394–407. ©2013 AACR.

Introduction

Breast cancer is the most common cancer in American women with an estimated 232,340 new cases of invasive and 64,640 new cases of noninvasive breast cancer this year alone. In 2013, 39,620 women were expected to die from breast cancer, and about 1 in 8 U.S. women would develop invasive breast cancer over the course of her lifetime (1). Breast cancer is frequently classified on the basis of hormone receptor status where 60% of premenopausal and 75% of postmenopausal cancers are estrogen receptor–positive (ER+; ref. 2). The ER acts as a master regulator of gene expression in breast cancer and promotes tumor progression via upregulating genes for proliferation and cell survival while down-regulating proapoptotic and tumor-suppressing factors (3, 4).

Tamoxifen is prescribed as an adjuvant therapy in early breast cancer (5). It improves overall survival, and is thought to have made a significant contribution to the reduction in breast cancer mortality over the last decades (6). Unfortunately, up to 50% of ER+/PR+ (ER/progesterone receptor–positive) tumors, 66% of ER+/PR− cases, and 55% of ER−/PR+ cases fail to respond or develop early resistance to tamoxifen despite an initial response and eventually relapse with more aggressive tumors (7–9). A greater understanding of the complex and multifactorial changes in the mechanism of tamoxifen action is needed to predict and prevent the emergence of resistance to endocrine therapy.

Much of the research investigating the mechanism of tamoxifen resistance has implicated various signaling cascades, such as EGFR/HER2 (10), PI3K/AKT (11), and stress-activated kinase/c-junNH2 terminal kinase pathways (12). Recently, a retrospective immunohistochemical study of primary tumors showed that overactivity of the AKT pathway was significantly associated with a reduced response to tamoxifen therapy (13). In another study, the overexpression of phosphorylated AKT or active AKT (pAKT) was detected in 58% of breast cancer samples, and survival analyses revealed that patients whose tumors were pAKT-positive were more prone to relapse with distant metastases (14). Previously, Kirkegaard and colleagues reported that

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patients who relapsed were more likely to have low AKT2 expression (15). Spears and colleagues reported that AKT1 activation was associated with a poor prognosis and decreased relapse-free survival in both ER+ and ER- breast cancers; whereas, AKT2 activation was associated with an increased relapse-free survival in ER+ but decreased relapse-free survival in patients with ER- breast cancer (16). High cytoplasmic levels of AKT1 and AKT3 were correlated with high pAKT, which is implicated in endocrine resistance and an increased incidence of metastatic tumors (17). However, such reports have not investigated the mechanism of AKT-induced tamoxifen resistance.

To study the role of AKT overexpression in tamoxifen resistance, we used tamoxifen-sensitive MCF-7 breast cancer cells, which have low levels of AKT. We transfected constitutively active AKT in these breast cancer cells and observed that these transfected cells, along with other breast cancer cells expressing high levels of endogenous AKT, exhibited tamoxifen-stimulated cell proliferation and enhanced cell motility. We used microarray gene expression profiling to identify the genes contributing to AKT-induced tamoxifen resistance. Functional analyses of candidate genes reveal that AKT alters important cellular processes such as cell proliferation, migration, metabolism, and signal transduction that may contribute to tamoxifen resistance in these cells. In this article, we demonstrate that ribonucleotide reductase M2 (RRM2), which promotes DNA synthesis and is suggested to be upregulated by estrogen in MCF-7 cells (18), is upregulated after tamoxifen treatment in AKT-expressing cells. In our study, inhibition of RRM2 by siRNA significantly inhibited tamoxifen-induced cell proliferation, inhibited the expression of DNA repair genes, and inhibited cell motility in motile AKT-expressing cells. As such, RRM2 may contribute to tamoxifen resistance and serve as a potential target to reverse tamoxifen resistance. To our knowledge, this is the first study that uses gene expression profiling in tamoxifen-resistant AKT-expressing breast cancer cells to investigate genes that may be involved in conferring resistance to endocrine therapy in breast cancer cells. Rational combination therapies are considered to be the most efficient strategy to combat cancer and to overcome drug resistance. Here, we studied the combination of two drugs, which have been suggested to cause tumor cell death by different mechanisms. Using comprehensive cell culture and in vivo models, we demonstrate for the first time that combining tamoxifen with the ribonucleotide reductase inhibitor didox (3,4-dihydroxybenzohydroxamic acid) potently kills AKT-expressing breast cancer cells (19, 20).

Materials and Methods

Cell culture and treatment
Late-passage MCF-7 cells were originally received from the American Type Culture collection (ATCC) and maintained in Advanced DMEM (Dulbecco’s Modified Eagle Medium) supplemented with 5% FBS, streptomycin, and penicillin. Cell media components were purchased from Life Technologies while 17β-estradiol and 4-hydroxy-tamoxifen were purchased from Sigma. For experiments, cells were washed with Hank’s balanced salt solution (HBSS) and kept in phenol red-free DMEM-F12 media supplemented with 5% charcoal-stripped serum (CSS) for 48 hours. For treatments, cells were then washed with HBSS and treated with 10-7 mol/L ethanol as vehicle control, 10-8 mol/L 17β-estradiol, 10-6 mol/L 4-hydroxy-tamoxifen, 10-7 mol/L 4-hydroxy-tamoxifen, or 10-8 mol/L 4-hydroxy-tamoxifen in phenol red-free, serum-free DMEM-F12 for 24 hours. Didox was synthesized and kindly provided by Dr. Howard Elford, Molecules for Health (Richmond, VA). All of the compounds were dissolved in 0.9% sterile saline solution, then filtered through a 0.45-μm syringe filter, and stored at 4°C in the dark for a maximum of 1 week.

RNA extraction and reverse transcriptase quantitative PCR
Total RNA was isolated using RNeasy according to the manufacturer’s protocol (Qiagen). Reverse transcriptase quantitative PCR (RT-qPCR) reactions were performed using SYBR green technology and primers are listed in Supplementary Table S1A. Fold change was determined using the comparative 2ΔΔCt method using the experimentally validated unaltered housekeeping gene RPL13A (23). For details, see Supplementary Materials and Methods.

Western blot analyses
Cells were disrupted in AKT lysis buffer (all components from Sigma), and Western blot analyses were performed and immunoblotted with pAKT(S473), AKT1, AKT2, AKT3, total AKT, BIM, ER-α, GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Cell Signaling Technology), and RRM2 (Sigma). For details, see Supplementary Materials and Methods.

Cell proliferation
Cell proliferation was measured using CellTiter 96 AQueous One Solution according to the manufacturer’s protocol (Promega). Briefly, 1,000 cells were plated in each well of a 96-well plate in phenol red-free, DMEM-F12 medium with 2% CSS. After 24 hours, cells were treated as mentioned above and treatment media was replenished on days 2, 4, and 6. At the end of days 1, 3, 5, and 7, the AQeous One Solution reagent was added, incubated for 1 hour, and measured at 490 nm. Results are shown as the mean ± SEM of three independent experiments done in triplicate.

Cell motility, migration, and invasion
A modified scratch assay was performed using cell culture inserts (ibidi) as per the manufacturer’s protocol using 2 × 104 cells per well in a 96-well plate. Cell migration and invasion experiments were carried out with the QCM 24-well Colorimetric Cell Migration Kit and
the Invasion Assay Kit (Chemicon) according to the manufacturer’s instructions. For details, see Supplemental Materials and Methods.

Microarray and data analysis
Parental and AKT3-overexpressing MCF-7 breast cancer cells were treated with $10^{-7}$ mol/L control, $10^{-6}$ mol/L 17β-estradiol, or $10^{-7}$ mol/L 4-hydroxy-tamoxifen using Agilent’s human Two-Color Microarray-based Gene Expression Analysis platform (G2519F) according to the manufacturer’s protocol. All microarray data have been deposited into the Gene Expression Omnibus (GEO) under the accession number of GSE34291 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34291; Supplementary Table S1B). Venn diagrams were generated using lists of differentially expressed genes. The relative expression of individual candidate genes in human breast tumor samples was determined using the Oncomine database (24). Oncomine output data was sorted to isolate “Breast carcinoma versus normal” associations using box-and-whisker plots. Independent correlation was confirmed using the TCGA (The Cancer Genome Atlas) database (Supplementary Fig. S1; ref. 25). For details, see Supplemental Materials and Methods.

siRNA-mediated suppression of RRM2
siRNA oligos targeting RRM2 (siRRM2) were designed and synthesized at Genetech Inc. and nontargeting siRNA and Dharmafect-1 were purchased (Dharmacon). Cells were transfected with siRNA by reverse transfection as per the manufacturers’ directions. Transfection efficiencies were evaluated relative to nontargeting control by RT-qPCR, and the suppression of RRM2 expression was sustained through day 7 for all breast cancer cell lines (Supplementary Fig. S5D).

Establishment of acquired tamoxifen-resistant cell lines
MCF7, T47D, HCC1428, and BT-483 were continuously exposed to increasing concentrations of tamoxifen (up to 5 μmol/L maximum). The resistant cell lines were maintained in continuous culture with this maximum dose of tamoxifen.

Colony formation assay
Parental and MCF-7 TamR, T-47D TamR, HCC1428 TamR, and BT483 TamR cells were cultured in 5% FBS phenol red–free DMEM. Briefly, cells were plated at a low density and later treated with tamoxifen and/or didox. Cells were allowed to grow for 10 days. Colonies were stained and quantified. Experiments were conducted in triplicate and data represented as mean ± SEM. For details, see Supplemental Materials and Methods.

Pathway-specific expression array
The human DNA damage signaling RT² Profiler PCR array (SABiosciences) was used to assess the impact of RRM2 reduction on the expression of 84 DNA damage genes according to the manufacturer’s instructions. Data shown represent the average of two replicates and were normalized for RPL13A levels by the $2^{-ΔΔCt}$ method.

Caspase activity and DNA damage assays
Caspase activity was determined by using a Caspase-Glo 6/8 Assay Kit (Promega), according to the manufacturer’s instructions. DNA damage was evaluated by using the HCS DNA Damage Kit according to the manufacturer’s protocol. The mean intensity from phosphoS139-gamma-H2AX and Image-it DEAD Green stain was determined.

Xenograft studies and immunohistochemical analyses
All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of the Pacific. AKT-overexpressing MCF-7 cells (4 × 10⁶ per site) were subcutaneously injected into the flank of ovariectomized nude mice (6 tumors per group). As indicated, mice were concurrently implanted with tamoxifen pellets (5 mg, Innovative Research of America) and injected with didox (425 mg/kg). For intraperitoneal injections, didox was dissolved in water, filtered, and injected fresh each day. At study termination, tumors were harvested and either stored at −80°C or fixed with 10% formalin. Tumors were processed for immunohistochemical staining (RRM2 and Ki-67) by the UC Davis Veterinary Medical Teaching Hospital. For details, see Supplemental Materials and Methods.

Results
Differential AKT isoform expression is observed in breast cancer tumors
To determine the clinical significance of AKT expression, we assessed the association of AKT isoform expression in normal and breast cancer patient samples using the Oncomine database. In a cohort of breast cancer tumors compared with normal breast tissue, we found significantly higher expression of AKT1 ($P = 0.000396$) and AKT3 ($P = 0.0015$) in tumors (Fig. 1A; ref. 26). We also found that AKT1 and AKT3 strongly correlate with higher-grade breast tumors (27). However, AKT2 did not significantly correlate with breast cancer or tumor grade in either study (Fig. 1B). In breast cancer patient samples, only AKT3 was significantly increased with recurrence ($P = 0.0015$). We observed no difference in AKT1 and AKT2 expression in these patient samples (Fig. 1C; ref. 26). Because we observed an in vitro reduction in the expression of the ER after AKT overexpression, we investigated whether the expression of specific AKT isoforms correlates with ER status. We observed that only AKT3 correlates with ER− status ($P = 0.002$; Fig. 1D; ref. 27).

Expression profile of ER-α and AKT isoforms in six breast cancer cell lines
Because both AKT1 and AKT3 are expressed in higher levels in many breast tumors and their expression correlates with higher-grade tumors, we chose the ER−, tamoxifen-sensitive, MCF-7 breast cancer cell line, which has low levels of both of these AKT isoforms, to study the

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contribution of AKT1 and AKT3 isoforms in tamoxifen resistance (Fig. 2B). We have previously overexpressed constitutively active AKT in MCF-7 breast cancer cells, which induced in vivo tumor growth and reduced in vitro cell death upon tamoxifen treatment (28). We now use this model system along with a panel of other breast cancer cell lines to investigate genes that are differentially expressed upon tamoxifen treatment in AKT-expressing breast cancer cells.

We examined parental, AKT1, and AKT3-overexpressing MCF-7 cells, along with MDA-MB-468, ZR-75-1, and MDA-MB-231 cells for ESR1 and AKT isoform expression (Fig. 2A and B). Parental MCF-7 and ZR-75-1 cells exhibited the highest expression of ESR1. Relative to parental cells, AKT1 and AKT3-overexpressing MCF-7 cells had both lower RNA expression and protein levels of the ER (Fig. 2). We observed that AKT1-overexpressing MCF-7, ZR-75-1, and MDA-MB-231 cells had significantly higher RNA expression.

Figure 1. AKT1 and AKT3 are highly expressed in patient breast cancer tumors and AKT3 expression correlates with recurrence and ER status. Box-and-whisker plot of AKT isoforms expression in normal versus breast carcinoma specimens (A), tumors of different grade (B), and tumors with respect to recurrence (C) and ER status (D) using Oncomine datasets (26, 27).
expression and higher protein levels of AKT1 than parental MCF-7 cells. Similarly, AKT3-overexpressing MCF-7 and MDA-MB-231 cells had the highest RNA expression and protein levels of AKT3. The highest levels of phosphorylated AKT (pAKTS473) were observed in the AKT1-overexpressing MCF-7, AKT3-overexpressing MCF-7, and MDA-MB-468 cells (Fig. 2C).

Tamoxifen induces cellular proliferation in AKT-expressing cells

To determine the effect of AKT expression on cell proliferation and tamoxifen sensitivity, cells were treated with control, estrogen, or three doses of tamoxifen. As expected, we observed estrogen-induced cell proliferation in the ER\(^++\) parental MCF-7 and ZR-75-1 breast cancer cells. In the parental MCF-7 cell line, which expresses low levels of both AKT1 and AKT3, we observed that tamoxifen inhibited cell proliferation but did not in the remaining cell lines (Fig. 3A). In addition, in the case of AKT1 and AKT3-overexpressing MCF-7 cells, all three doses of tamoxifen significantly stimulated cell proliferation (Fig. 3A).

Under vehicle control treatment, all of the cell lines expressing high levels of AKT, namely AKT1 and AKT3-overexpressing MCF-7, MDA-MB-468, ZR-75-1, and MDA-MB-231 cells as compared with parental MCF-7 cells (Fig. 3B). To determine the additive effect of tamoxifen on AKT-induced cell proliferation, fold proliferation was determined by comparing tamoxifen with control treatment for each cell line. By day 7, tamoxifen induced significantly higher-fold proliferation in cell lines with high AKT, namely AKT1 and AKT3-overexpressing MCF-7, MDA-MB-468, ZR-75-1, and MDA-MB-231 cells as compared with their own controls and tamoxifen induced significantly lower cell proliferation only in parental MCF-7 breast cancer cells (\(P < 0.05\); Fig. 3C). Because tamoxifen even had proliferative effects on the ER\(^-\) cells lines, this suggests that these cell lines may actually have very low levels of ER-\(\alpha\), as is evident in Fig. 2C and D.
AKT overexpression induces cell motility

Because expression of AKT1 and AKT3 strongly correlates with higher breast tumor grades (Fig. 1B), we wanted to determine whether AKT1 and AKT3 would result in increased cell motility as typically observed in higher-grade breast cancers. As such, we investigated the effect of AKT expression on in vitro cell motility in the six breast cancer cell lines by performing a modified scratch assay. We observed that parental MCF-7, MDA-MB-468, and ZR-75-1 cells did not display any significant cell motility over 20 hours. Conversely, AKT1 and AKT3-overexpressing MCF-7 cells, as well as MDA-MB-231 cells (which express high levels of AKT1 and AKT3) were significantly more motile as compared with parental MCF-7 cells (Fig. 4).

Differential gene analysis reveals that AKT alters genes involved in important molecular processes

Dye-swap, two-color, gene expression analysis was performed on RNA from parental and AKT-overexpressing MCF-7 cells treated with vehicle control, estrogen, or tamoxifen. To determine the role of the genes differentially expressed in AKT-overexpressing cells, we generated a list of genes that were significantly expressed on two or more microarrays and classified these genes according to their biologic functions using Ingenuity Pathway Analysis (IPA) software (Supplementary Tables S2 and S3). Most of these differentially regulated genes are involved in important molecular processes.
molecular processes such as cell proliferation and cell motility.

**Tamoxifen alters cell proliferation and cell motility genes in AKT-expressing cells**

Because we observed tamoxifen-induced cell proliferation in AKT-expressing cells, we wanted to determine whether such treatment would also alter the expression of genes related to cell proliferation. To identify genes that may contribute to the altered hormonal responsiveness that we observed in AKT-overexpressing cells, we compared control, estrogen, or tamoxifen-treated parental with AKT-overexpressing MCF-7 cells (MC:AC, ME:AE, and MT:AT) using Venn diagrams of these three arrays (Supplementary Fig. S2A and Supplementary Table S2).

Because estrogen induced growth in parental MCF-7 (MC:ME) and tamoxifen induced growth in AKT-expressing breast cancer cells (MT:AT), we compared these two arrays using Venn diagrams and observed 10 common genes, including GAL, H19, RRM2, MME, and PTPRJ (Supplementary Fig. S2B). This may suggest that tamoxifen in AKT-overexpressing cells induces cell proliferation through a similar set of genes as does estrogen in parental MCF-7 cells.

Using our list of differentially expressed genes from all seven microarray arrays, we performed IPA analysis for each microarray to group genes according to gene networks of top
functions (Supplementary Table S3). In the MT:AT microarray, cell proliferation and cell motility were the top two networks of genes. In the MC:ME microarray, cell proliferation and gene expression were the top two networks of genes. As expected, the top network of genes for tamoxifen-treated parental MCF-7 cells (MC:MT microarray) was associated with cell death, as tamoxifen inhibits cell proliferation in parental MCF-7 cells.

To gain insight into the networks of genes involved in the tamoxifen-induced cell proliferation of AKT-overexpressing cells, we used IPA to generate canonical pathways impacted by the differentially expressed genes on the MC:ME (Supplementary Fig. S2C) and MT:AT microarrays (Supplementary Fig S2D). Our network analysis of differentially expressed genes from these two microarrays revealed that both had cell proliferation as a top network with seven shared genes in this category. For the differentially expressed genes on the MT:AT microarray, estradiol was identified by IPA as the central node in the cell proliferation network (Supplementary Fig. S2C). However, for the differentially expressed genes on the MT:AT microarray, NFκB was determined to be the central node in the cell proliferation network (Supplementary Fig. S2D).

Validation of gene expression data by RT-qPCR analysis in a panel of breast cancer cells

From Supplementary Table S2, 10 representative genes were selected for validation by RT-qPCR in our parental and AKT-transfected MCF-7 cells along with the other breast cancer cells that we evaluated in Figs. 2–4. RT-qPCR data are presented as the mean fold change relative to the indicated control of three independent experiments done in triplicate. The fold changes that we observed by RT-qPCR were in direct agreement and quite consistent with our microarray data for all selected genes (Supplementary Table S4). CGA, CPE, GAL, H19, and RRM2 were upregulated and validated by RT-qPCR (Supplementary Fig. S3A and Fig. 5A). One gene in particular, RRM2, was upregulated more than 2-folds with estrogen treatment in parental MCF-7 cells. Interestingly, this same game RRM2 was upregulated by 2 to 9 fold with tamoxifen treatment in AKT-expressing breast cancer cell lines as compared with tamoxifen-treated parental MCF-7 cells (Supplementary Table S4). ASAM, CGNL1, MME, PIM1, and PTPRJ were downregulated and validated by RT-qPCR (Supplementary Fig. S3B).

Differentially altered genes correlate with clinical breast carcinoma

We assessed the association of these 10 selected genes in normal and breast cancer samples (Supplementary Fig. S4). Our analysis revealed that all 10 selected genes are differentially expressed in breast carcinoma as compared with normal breast tissue (P<0.02) with fold expression ranging from 2.7 to 7.7 for upregulated genes and 2.7 to 6.0 for downregulated genes (27, 29–32). Specifically, RRM2 was significantly associated with higher expression in breast carcinoma by 5.9-fold with a P value of 0.00000637 (Fig. 5B).

RRM2 knockdown reverses AKT-induced tamoxifen resistance in breast cancer cells

To validate our array data, RRM2, a highly upregulated gene in tamoxifen-treated AKT-expressing breast cancer cells, was carried forward for functional analysis. We examined the effect of RRM2 knockdown on AKT-induced tamoxifen resistance. We transfected our panel of six breast cancer cells with two optimized RRM2 siRNAs (siRRM2#1 and siRRM2#2) and a nontargeting control oligo. Cell proliferation studies were performed in control and tamoxifen-treated (10−6 mol/L) breast cancer cells over seven days as previously described. In tamoxifen-resistant breast cancer cells with high levels of AKT, we observed that RRM2 knockdown resulted in a 43% to 60% reduction in tamoxifen-treated cell proliferation in AKT-expressing cells as compared with only 3% reduction in parental MCF-7 cells (Fig. 5C). Treatment with siRRM2 induced only a minor reduction in cellular proliferation under control treatment (Supplementary Fig. S5C). Knockdown expression of RRM2 was validated by RT-qPCR as well as Western blot analysis 48 hours after transfection (Supplementary Fig. S5A and S5B), and knockdown efficiencies remained high over seven days as validated by RT-qPCR (Supplementary Fig. S5D).

RRM2 knockdown inhibits cell motility in six breast cancer cell lines

To evaluate the role of RRM2 in cell motility, we performed a modified scratch assay upon transfection of siRRM2 or a nontargeting control in six breast cancer cell lines. Parental MCF-7, MDA-MB-468, and ZR-75-1 did not display any significant difference in motility between nontargeting control and siRRM2 knockdown. However, transfection with siRRM2 in AKT1, AKT3, and MDA-MB-231 cells abolished the motile phenotype that was previously observed (Figs. 4, Fig. 5D, and Supplementary Fig. S5E). Moreover, we observed a significant reduction in cell migration and cell invasion through Boyden chamber–based assays with siRRM2 as compared with nontargeting controls (Fig. 5E and F).

RRM2 regulates DNA damage and apoptotic genes upon tamoxifen treatment

Because RRM2 plays an important role in DNA repair by ensuring continuous dNTP supply (33) and regulating apoptosis (34), RRM2 inhibition may exacerbate DNA damage. We profiled expression of 84 genes involved in DNA damage signaling by the RT2 profiler PCR assay on tamoxifen-treated nontargeting versus siRRM2-transfected AKT3-overexpressing MCF-7 cells. Using a fold-difference cutoff of greater than 2.0, we observed that inhibiting RRM2 repressed the expression of key DNA repair enzymes, and induced the expression of proapoptotic factors like BIM and BAX (Fig. 6A). Because we observed that double-stranded DNA repair enzymes were also inhibited in our PCR array, we tested whether inhibiting RRM2 alters the S139 phosphorylation of the histone H2AX (gamma-H2AX), a marker for DNA damage (35). Moreover, we observed that tamoxifen induced DNA damage in
parental MCF-7 cells but not in AKT-expressing cells. Yet, in tamoxifen-treated AKT-expressing cells, we observed that inhibition of RRM2 decreased the phosphorylation of gamma-H2AX, suggesting that inhibition of RRM2 inhibits DNA repair (Fig. 6B), and these tamoxifen-treated cells are now undergoing apoptosis as indicated by the activation of caspases (Fig. 6C and D) and quantified by Image-iT DEAD Green stain (Fig. 6E). Western blot analysis confirmed the induction of BIM upon inhibition of RRM2 expression in tamoxifen-treated AKT-expressing breast cancer cells as compared with parental MCF-7 cells as observed in the PCR array (Fig. 6F).

**Inhibition of RRM2 reverses AKT-induced tamoxifen resistance in breast cancer cells**

In addition, we tested the small-molecule inhibitor of ribonucleotide reductase activity didox (36), on tamoxifen-resistant AKT-expressing cells. Cell proliferation assays were performed on AKT-expressing breast cancer cells treated with didox in the presence or absence of tamoxifen. As shown in Figure 5, RRM2 inhibition reverses tamoxifen-induced cell proliferation and cell motility. A, RRM2 is upregulated in tamoxifen-treated AKT-expressing breast cancer cells as confirmed by RT-qPCR. Fold change is shown relative to control-treated parental MCF-7 cells. B, RRM2 is overexpressed in breast carcinoma tumor samples. C, RRM2 knockdown reverses AKT-induced tamoxifen resistance in breast cancer cells. Cell lines were treated as indicated with nontargeting control (NT C), siRRM2#1 control, siRRM2#2 control, nontargeting tamoxifen treatment (NT T), siRRM2#1 tamoxifen, or siRRM2#2 tamoxifen from 0 to 7 days. D, RRM2 knockdown inhibits cell motility in six breast cancer cell lines. Percent open area was plotted against time for six breast cancer cell lines. Knockdown of RRM2 inhibits (E) cell migration and (F) invasion in AKT-overexpressing breast cancer cell lines. Error bars, mean ± SEM of three independent experiments done in triplicate. Comparisons between groups were made by two-sample t tests; *, P < 0.05; **, significant difference between tamoxifen-treated nontargeting versus siRRM2-treated breast cancer cell lines.
performed after treatment with control, tamoxifen, didox, or the combination of didox and tamoxifen in our panel of breast cancer cell lines. Similar to knockdowns of RRM2, didox treatment alone did not decrease cell proliferation, but didox in combination with tamoxifen significantly reduced in vitro cell proliferation in AKT-expressing cells (Fig. 7A). Previously, we reported that tamoxifen stimulates tumor growth of AKT-overexpressing MCF-7 breast cancer in vivo (28). We used this model to test the ability of didox to inhibit in vivo tamoxifen-induced cell proliferation. AKT-overexpressing cells were injected into the hind flanks of nude female mice treated with control, tamoxifen, or tamoxifen and didox. As expected, we observed that tamoxifen caused a 3-fold increase in the size of the tumors formed. However, the RRM2 inhibitor didox blocked the tamoxifen-induced increase in AKT-overexpressing xenograft tumors (Fig. 7B). Throughout the study, no changes in body weight were observed with didox treatment. In animals treated with didox and tamoxifen as compared with no treatment or tamoxifen alone, RRM2 levels were lower in tumor xenografts as measured by RT-qPCR, Western blot, and immunohistochemistry analyses of tumor xenografts (Fig. 7C–E).

**RRM2 is overexpressed in acquired tamoxifen-resistant breast cancer cell lines**

To investigate whether RRM2 is also overexpressed in acquired tamoxifen resistance, we established the resistant subcell lines MCF-7 TamR, T-47D TamR, HCC1428 TamR, and BT483 TamR from their ER⁺ breast cancer parental cells by selecting them in the presence of 5-μmol/L tamoxifen for 2 months. Compared with the parental cell lines, TamR cell lines exhibited insensitivity to tamoxifen (Fig. 7F). Western blot analysis revealed that the expression

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**Figure 6.** Inhibiting RRM2 represses DNA repair enzymes and induces expression of proapoptotic factors upon tamoxifen treatment. A, fold change of selected genes as measured by DNA damage PCR array is shown as repressed (negative) or induced (positive) in tamoxifen-treated nontargeting as compared with siRRM2-transfected AKT3-overexpressing MCF-7 cells. B, quantification of DNA damage as measured by the intensity of gamma-H2AX staining normalized to control nontargeting cells for each of the breast cancer cell lines. Tamoxifen treatment of siRRM2 AKT-overexpressing cells causes apoptosis as measured by fold induction of (C) caspase-8 activity, (D) caspase-6 activity, (E) quantification of the cytotoxicity by Image-iT DEAD Green cells, and (F) Western blot showing induction of the proapoptotic factor BIM. Error bars, mean ± SD of triplicate samples. Comparisons between groups were made by two-sample t tests; *, P < 0.05.
level of RRM2 was increased in TamR subcell lines (Fig. 7G). Moreover, inhibiting RRM2 by didox treatment alone did not significantly reduce proliferation in the tamoxifen-sensitive parental cell lines but did have some effect in the MCF-7, T47D, and HCC1428 TamR subcell lines. In all of the TamR subcell lines, the combination of didox and tamoxifen significantly reduced cell proliferation as measured in the cell proliferation assay (Fig. 7H).

Discussion

In this study, we show that AKT overexpression alone in tamoxifen-sensitive, ER⁺ breast cancer cells is sufficient to confer tamoxifen-resistant cell proliferation. To identify genes that may be involved in AKT-induced tamoxifen resistance, we used microarray analysis to identify differentially expressed genes. Functional analysis revealed that two main biologic processes are altered: cell proliferation and cell...
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Motility (Supplementary Table S2), supporting our in vitro observations seen in Akt-expressing cells. Because expression of both AKT1 and AKT3 are significantly greater in patient breast tumors (Fig. 1) and because tumors exhibit greater cell proliferation and motility than normal cells, it was not surprising that these same differentially expressed genes also strongly correlated with breast carcinoma as compared with normal breast tissue in existing tumor cohorts, further validating our array data (Fig. 5B and Supplementary Fig. S4).

From our IPA analysis, estrogen and NFκB were identified as the central nodes in MC:ME and MT:AT microarrays, respectively. Because AKT is known to regulate gene expression via NFκB transcriptional activity and as enhanced NFκB transcriptional activity has been associated with tamoxifen-resistant breast cancers (37), we used the transcription factor binding predictive tool, Genomatix’s MatInspector, to analyze our 10 selected genes for NFκB binding sites and determined that eight genes (CGA, GAL, H19, RRM2, CGNL1, MME, PIM1, and PTPRJ) indeed had NFκB transcription factor binding sites near their transcription starting site. One of these representative genes, RRM2, which was induced in both the MC:ME and MT:AT microarrays was functionally validated after RRM2 inhibition was shown to reverse AKT-induced tamoxifen resistance.

To our knowledge, our study is the first one to demonstrate that overexpression of RRM2 may contribute to Akt-induced tamoxifen resistance in breast cancer cells. Overexpression of RRM2 has been reported to markedly enhance the cellular transforming potential of various oncogenes and to increase the malignant potential of transformed cells (38). RRM2 overexpression has also been associated with gemcitabine resistance (39). Recently, Huang and colleagues reported RRM2 overexpression in tamoxifen-resistant tumors using a bioinformatics approach (40). Here, we have identified RRM2 as one of many genes that was upregulated upon tamoxifen treatment in our in vitro model of AKT-overexpressing breast cancer cells. In addition, we found a strong correlation between RRM2 expression in clinical breast carcinoma as compared with normal breast tissue.

The finding that RRM2 inhibition restores the sensitivity of Akt-expressing breast cancer cells to tamoxifen implies that RRM2 might be an important mediator in AKT-induced tamoxifen resistance. The AKT regulation of RRM2 has not been previously reported, but it could be through NFκB transcription factor binding sites as mentioned above. Classically, tamoxifen is a selective ER modulator that binds to ER-α and recruits corepressors and inhibits ER-regulated gene synthesis (5). Apart from this classic ER action, tamoxifen also induces DNA damage and apoptosis in ER+ cell lines (41). However, Akt-expressing cells seem to upregulate RRM2 expression and are protected from tamoxifen-induced cell death. In our study, we found that inhibiting RRM2 induces phospho-S139 gamma-H2AX a well-known marker of DNA damage, activates caspases, and subsequently induces apoptosis in tamoxifen-resistant cells. Our PCR array of siRRM2-transfected tamoxifen-treated AKT-overexpressing cells revealed that inhibition of RRM2 downregulates the expression of key repair enzymes and induces the expression of proapoptotic genes (Fig. 6A). Thus, it is possible that overexpression of RRM2 in AKT-expressing cells may lead to increased DNA repair and protection from tamoxifen-induced apoptosis in tamoxifen-treated cells.

Treatment of malignant cells with the RRM2 inhibitor didox can induce a wide range of anticancer effects, including reduced cell proliferation, cell-cycle arrest, and apoptosis (42). Didox was clinically evaluated decades ago, and the phase II trial in patients with advanced breast cancer did not support its use as a single agent at the given dose (43). Because didox monotherapy does not seem to be effective against breast tumors, its full therapeutic potential may be better realized in combination with other anticancer agents. In this study, we provide several lines of evidence to support combining inhibition of RRM2 with tamoxifen therapy. First, the combination of tamoxifen and didox reduced cell proliferation and induced DNA damage and apoptosis in AKT-expressing cell lines. Second, the combination also inhibited cell proliferation in acquired tamoxifen-resistant breast cancer cell lines. Finally, this combination therapy inhibited tumor growth in our in vitro model of tamoxifen resistance. These findings further support our hypothesis that inhibition of RRM2 may be a useful approach in reversing tamoxifen resistance.

In summary, our studies demonstrate that AKT may be a critical factor in promoting resistance to tamoxifen. We have observed that AKT overexpression results in increased in vitro cell proliferation and motility and that tamoxifen treatment of these cells further induces cellular proliferation. These results may provide a molecular basis for our finding that AKT1 and AKT3 expression levels are significantly higher in patient breast tumors versus normal breast samples and that the expression of AKT1 and AKT3 correlate with breast tumor grade. In addition, AKT3 expression correlates with recurrence and ER status in patients with breast cancer. We have identified genes that are differentially expressed upon hormonal treatment in AKT-overexpressing breast cancer cells by microarray analysis. Together, our findings suggest that AKT-regulated genes may be used as potential biomarkers and may serve as potential targets for drug discovery and for reversing resistance. Specifically, this is the first demonstration that RRM2 may contribute to tamoxifen resistance and serve as a potential target to restore tamoxifen sensitivity in breast cancer cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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