Comparison of tamoxifen and letrozole response in mammary preneoplasia of ER and aromatase overexpressing mice defines an immune-associated gene signature linked to tamoxifen resistance

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Response to breast cancer chemoprevention can depend upon host genetic makeup and initiating events leading up to preneoplasia. Increased expression of aromatase and estrogen receptor (ER) is found in conjunction with breast cancer. To investigate response or resistance to endocrine therapy, mice with targeted overexpression of Esr1 or CYP19A1 to mammary epithelial cells were employed, representing two direct pathophysiologic interventions in estrogen pathway signaling. Both Esr1 and CYP19A1 overexpressing mice responded to letrozole with reduced hyperplastic alveolar nodule prevalence and decreased mammary epithelial cell proliferation. CYP19A1 overexpressing mice were tamoxifen sensitive but Esr1 overexpressing mice were tamoxifen resistant. Increased ER expression occurred with tamoxifen resistance but no consistent changes in progesterone receptor, pSTAT3, pSTAT5, cyclin D1 or cyclin E levels in association with response or resistance were found. RNA-sequencing (RNA-seq) was employed to seek a transcriptome predictive of tamoxifen resistance using these models and a second tamoxifen-resistant model, Brca1 deficient/Tpr53 haploinsufficient mice. Sixty-eight genes associated with immune system processing were upregulated in tamoxifen-resistant Esr1- and Brca1-deficient mice, whereas genes related to aromatic compound metabolic process were upregulated in tamoxifen-sensitive CYP19A1 mice. Interferon regulatory factor 7 was identified as a key transcription factor regulating these 68 immune processing genes. Two loci encoding novel transcripts with high homology to human immunoglobulin lambda-like polypeptide 1 were uniquely upregulated in the tamoxifen-resistant models. Letrozole proved to be a successful alternative to tamoxifen. Further study of transcriptional changes associated with tamoxifen resistance including immune-related genes could expand our mechanistic understanding and lead to biomarkers predictive of escape or response to endocrine therapies.

Introduction

Targeted overexpression of estrogen receptor 1 (Esr1) and cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1) to mammary epithelial cells of genetically engineered mouse (GEM) model results in overexpression of their respective proteins, estrogen receptor (ER) alpha and aromatase, followed by development of mammary hyperplasia and invasive cancer (1–3). These GEM models represent two direct pathophysiologic interventions in estrogen pathway signaling, the first at the receptor level and the second involving ligand. Mammary-targeted CYP19A1 expression increases mammary-localized aromatase activity but does not increase circulating estrogen levels and is sufficient to promote development of hyperplastic alveolar nodules (HANs) and cancer (3). Both overexpression of Esr1 and CYP19A1 in mammary epithelial cells increase expression levels of progesterone receptor (PGR) and phosphorylated insulin growth factor receptor, components of a high ‘ER activity profile’ suggested to be a criterion for selection of an aromatase inhibitor over tamoxifen for treatment of breast cancer (4). Esr1 overexpressing mice show intrinsic-type resistance to tamoxifen with development of mammary cancers on first exposure (5). GEM models lacking expression of full-length breast cancer 1, early onset (Brca1) conditionally targeted to mammary cells coupled with germ-line Tpr53 haploinsufficiency (Brca1 KO) also demonstrate tamoxifen resistance (6). BRCA1, more widely known for its role in DNA repair, has the capacity to repress ER function (7) and loss of BRCA1 function results in a mammary environment with increased sensitivity to estrogen in vivo (8,9). GEM overexpressing CYP19A1 treated with letrozole or mifepristone show significantly lower levels of hyperplasia but this is not found following IC1182,780 exposure (3).

Tamoxifen is a Food and Drug Administration-approved drug for breast cancer chemoprevention in both pre- and postmenopausal women; however, some treated women still develop ER+ or ER− breast cancer (10). Development of tamoxifen resistance in ER+ breast cancers is a well-recognized clinical challenge. Overexpressed molecules linked to tamoxifen resistance include cyclin D1 (11), cyclin E (12), signal transducer and activator of transcription (STAT) 3 (13) and STAT5 (14). Aromatase inhibitors, such as exemestane and letrozole, with use limited to postmenopausal women, are effective in reducing mammographic density and invasive breast cancer (15,16) and are being investigated as chemopreventives for women carrying BRCA1/2 mutations (17). There is a clinical need to identify women who are less or more likely to respond to available anti-hormonal agents (18–21) as diverse initiating events in breast cancer may respond differently to these agents.

Tissue transcriptomes are defined using high-throughput RNA-sequencing (RNA-seq) (22). Polyadenylated RNA, isolated from tissue and converted to complementary DNA for deep sequencing resulting in millions of short reads for reference genome mapping, yield an unbiased approach for evaluating differences in tissue transcriptomes. Here, genes that were significantly up- or downregulated in each of the three GEM models compared with wild-type (WT) mice were identified and gene expression patterns between tamoxifen-sensitive and tamoxifen-resistant models compared to detect differentially expressed genes (DEGs) and pathways.

Abbreviations: DEG, differentially expressed gene; DH, dactyl hyperplasia; ER, estrogen receptor; GEM, genetically engineered mouse; HAN, hyperplastic alveolar nodule; IFN, interferon; IFR, interferon regulatory factor; IGLL, immunoglobulin lambda-like polypeptide; IHC, immunohistochemistry; MCF-7, Michigan Cancer Foundation (MCF)-7; PGR, progesterone receptor; RNA-seq, RNA-sequencing; STAT, signal transducer and activator of transcription; TFBS, transcription factor binding site; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; WT, wild-type.

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interferon (IFN) regulatory factor (IRF) family of proteins have been reported to impact both progression and regression of malignant disease. In vitro continuous low exposure to IFN beta and activation of downstream genes including IRF9 leads to resistance to DNA damage and increased cell survival (27). IRF4 mediates a pathway suppressing cisplatin-induced apoptosis in vitro (28). High IRF7 expression in breast cancer correlates with longer metastasis-free survival (29), whereas loss of IRF5 is linked to invasiveness (30). IRF1 can restore sensitivity to IC182,780 in vitro (31). Immune signatures in different types of breast cancer are being studied for their predictive value in prognosis and therapeutic response (32).

GEM models are preclinical tools used to test how specific genetic interventions influence disease pathophysiology and treatment response. Well-defined genetic interventions can be introduced into GEM, whereas the genetics of disease pathophysiology are more challenging to define in human populations. HANs and ductal hyperplasia (DH) are mammary gland preneoplastic lesions correlating with increased risk of invasive mammary cancer development in mice (2,3,5,6,8) similar to histological abnormalities in women at elevated risk for breast cancer (33,34). In Er1 and CYP19A1 overexpressing mice, DH can be detected as early at 4 months of age, whereas HANs appear later, by 8 months of age (1,35).

Here, the aromatase inhibitor letrozole was more effective than tamoxifen at reducing rates of mammary epithelial cell proliferation and preneoplasia in mouse models with mammary-targeted overexpression of either ER or aromatase. Letrozole was an effective alternative to tamoxifen for reduction of preneoplasia in tamoxifen-resistant Er1 overexpressing mice. Tamoxifen resistance in Er1 overexpressing and BrCA1 KO mice was correlated with the presence of a gene signature indicating immune activation that included upregulation of If7 and downstream genes.

Materials and methods

Mouse models and treatment with tamoxifen and letrozole

At 10 months of age, cohorts of C57Bl/6 nulliparous female conditional tetra-cycline-regulated mammary-targeted Er1 (ER) overexpressing [tet-cycline-operator (tet-op)-Er1] Mouse Mammary Tumor Virus-reverse tetracycline Transactivator (MMTV-rtTA) and CYP19A1 (Aromatase) overexpressing (tet-op-CYP19A1(I192V)) (1,3) were implanted with slow release pellets (Innovative Research, Novi, MI) containing tamoxifen (25mg/60 day release), letrozole (2.5mg/60 day release) or maintained as controls without pellet placement [Er1 (n = 8 tamoxifen, n = 8 letrozole, n = 6 control); CYP19A1 (n = 12 tamoxifen, n = 11 letrozole, n = 8 control)]. Mammary glands from untreated nulliparous C57Bl/6 12-month-old Er1 (n = 3), CYP19A1 (n = 3), Brca1(fl11/fl11/MMTV-Cre/p53+/-) KO (n = 2) and non-transgenic WT mice (n = 1) were used for RNA-seq analyses. Preneoplastic and adenocarcinoma mammary tissues were obtained from untreated 12-month-old Brca1 KO mice (n = 6) and WT mice (n = 4). Mice were necropsied at age 12 months on one inguinal mammary gland processed for whole mount, one inguinal gland formalin fixed for histological studies and thoracic glands flash frozen at −20°C for molecular studies. Mice were maintained in barrier zones, generated from breeding colonies, weaned before puberty and placed in single-sex sterilized ventilated cages with corn cob bedding (1–4 mice per cage) with ad libitum access to water/chow under 12 h dark/light cycles at Georgetown University. Er1 and CYP19A1 KO mice were fed doxycycline-containing mouse diet (Bio-Serv, Frenchtown, NJ) from gestation main to timed pregnancy (tetracycline gene expression and Brca1(fli11/MMTV-Cre/p53+/-); and WT mice received irradiated Picolob rodent diet 20 (5053) (Labdiet, St Louis, MO). Mouse husbandry and euthanasia was performed in accordance with institutional and national guidelines under an animal protocol approved by the Georgetown University Animal Care and Use Committee.

Histological and immunohistochemical analyses

HANs were identified and counted on whole mounts of inguinal mammary glands fixed in Carnoy’s solution and stained in carmine alum (2). DH prevalence, Ki67, pSTAT5, and pSTAT3, were assessed using the Coding-Potential Assessment Tool (<0.44) and DNA damage in Brca1 KO compared with WT mice.

Gene ontology, network analyses, motif analysis and coding potential assessment

Identified DEGs were analyzed using BiNGO with default parameters (38) to infer molecular statistics of 2-fold repression or activation of DEGs, see Supplementary Table I, available at Carcinogenesis Online. For the gene ontology and pathway annotation network analysis, 68 upregulated genes in both Er1 and Brca1 KO but not CYP19A1 were analyzed with ClueGO with default parameters (Supplementary Table II, available at Carcinogenesis Online) (39). GeneMANIA and MCODE defined core genes (highly interconnected genes) among the 68 genes according to available databases (40,41). All the analyses were performed using the Cytoscape network analysis platform (42). For motif analysis, the software tool Pscan was used to identify overrepresented transcription factor binding sites (TFBSSs) on the promoter regions (−1 kb upstream to transcription start site) of the 68 upregulated genes using the JASPAR motif database (43,44). The top TFBSSs were defined by relative P value. Two highly relevant loci encoding several non-coding RNAs were identified in the Cufflinks transcription. To distinguish coding and non-coding RNAs, coding potential of the transcripts was assessed using the Coding-Potential Assessment Tool (45). Non-coding RNAs were defined with the optimized cutoff value for mouse (<0.44). The
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INTERFOLME database (http://interferome.its.monash.edu.au/interferome/) was used to identify genes regulated by IFN types I and II and locations.

Cell culture, RNA interference, reverse transcriptase–PCR and western blot analyses

Michigan Cancer Foundation (MCF)-7 cells (from Marvin Rich, Michigan Cancer Foundation, Detroit, MI), maintained in the Lombardi Cancer Center Tissue Culture Shared Resource, were last authenticated 22 September 2010 (Cell ID™ System, Promega, Madison, WI). Proliferating, subconfluent monolayers of MCF-7 cells were seeded at a density of 300,000 cells per well into 6-well tissue culture plates. Trilencer-27 Universal Scrambled Negative Control siRNA and IRF7 siRNA (three unique 27mer siRNA duplexes—2 nmol each (Locus ID 3665) (SR30245, Origene, Rockville, MD)) were transfected into cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions and 24 and 96 h later, cells were lysed in modified radioimmunoprecipitation assay buffer supplemented with Complete Mini protease inhibitor cocktail tablets (Roche, Mannheim, Germany), sonicated, clarified by centrifugation and concentration measured (Pierce 660 nm Protein Assay Reagent, Thermo Scientific) for western blot studies or lysed and total RNA extracted using an RNasey Mini Kit (QIAGEN, Gaithersburg, MD) for reverse transcription-PCR. Proteins were size fractionated by polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Non-specific binding was blocked by incubation with Tris-buffered saline containing 5% powdered milk and 1% Triton X-100 for 1 h at room temperature. Membranes were incubated with primary antibodies (IRF7, 4920, Cell Signaling Technology; PPAR14, sc-377150, Santa Cruz Biotechnology; IRGM1, ab69495, Abcam, Cambridge, MA; GBP7, E-20, Santa Cruz Biotechnology, actin, sc-1616, Santa Cruz Biotechnology) at 1:1000 dilution in Tris-buffered saline containing 1% Triton X-100 at 4°C overnight, followed by incubation with polyclonal horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution) for 1 h at room temperature, visualized by chemiluminescence (HyGLO Chemiluminescent HRP Antibody Detection Reagent, Denville Scientific, South Plainfield, NJ or SuperSignal West Pico ECL, Thermo Scientific) and ImageJ (http://image.nih.gov/) used to quantify TIFFF images (Epson Perfection 2400 PHOTO, Long Beach, CA). IRF7, PPAR14, IRGM1 and GBP7 signals were normalized to actin signals obtained from the same membrane and relative expression levels determined at the different time points/conditions. Mean and standard error of the mean were determined for each protein/condition/time point (n = 3, GraphPad Prism). RNA concentration was measured (Nanodrop, Thermo Scientific), complementary DNA prepared from 1 μg total RNA (iScript™ Reverse Transcription Supermix Kit, Bio-Rad, Hercules, CA) and PCR (GoTaq® Green Master Mix, Promega) performed to detect IRF7 (two primer sets): CTGGGACACTGTGTTCAACACCT and GTTGTGAATGAGGCTTGTAGG (1, 130 bp). CCACTTCTTCACCACGAGCAGCAGAAGGCTTGTAGGTA (#2, 100 bp), ACTB: CCTTGGCAATGCAGGGGAC and ACAGAGGTCGGCTGTTT (100 bp), GAPDH: AATGAACCATTTCAAATCAG and AAAGTGGATCGAAGAGGCTGTCG (100 bp) [30 cycles, 1m, 95°C; 1m, 57°C (h3, ACTB), 1m, 55°C (GAPDH), 3m, 72°C] with visualization (Bio-RAD UNIVERSAL, HOOD II, Bio-Rad) by ethidium bromide staining after electrophoresis on agarose gels.

Results

Letrozole was more effective than tamoxifen in reducing HANS and mammary epithelial cell proliferation in Esr1 and CYP19A1 overexpressing mice

Mammary preneoplasia includes both larger HANS demonstrable on mammary gland whole mounts at low magnification and DH detectable on hematoxylin and eosin-stained sections at higher magnification. At 12 months of age, both lesions were present in untreated Esr1 and CYP19A1 overexpressing mice with 67% of Esr1 overexpressing mice demonstrating HANS and 40% showing DH, whereas prevalence in CYP19A1 overexpressing mice were 60 and 31%, respectively (Figure 1A–C). HAN prevalence (P < 0.05, Fisher’s exact) and number/gland (P < 0.05, t-test, unpaired, one-tailed) were reduced to undetectable levels in both mouse models following 2 months of letrozole treatment initiated at 10 months of age. In contrast, 2 months of tamoxifen treatment reduced HAN prevalence (14%, P < 0.05, Fisher’s exact) and number/gland (0.1 ± 0.1, P < 0.05, t-test, unpaired, one-tailed) only in CYP19A1 overexpressing mice, but not Esr1 overexpressing mice (HAN prevalence 56%; HAN number/gland 0.8 ± 0.3) (Figure 1A and B). Both letrozole and tamoxifen significantly reduced DH prevalence in both models (Esr1 letrozole: none detected; tamoxifen: 12%; CYP19A1 letrozole: none detected; tamoxifen: 9%; P < 0.05, Fisher’s exact, all comparisons to control) (Figure 1C). Proliferative indices assessed by Ki67 IHC were significantly reduced in letrozole-treated (Esr1 control: 10.1 ± 4.5 versus letrozole: 2.3 ± 1.1 CYP19A1 control: 8.4 ± 4.7 versus letrozole: 1.4 ± 0.6, P < 0.05, Mann–Whitney, unpaired, one-tailed) but not tamoxifen-treated Esr1 and CYP19A1 overexpressing mice (Esr1 tamoxifen: 6.4 ± 1.5 CYP19A1 tamoxifen: 5.7 ± 0.9) (Figure 1D). Apoptotic indices measured using a TUNEL assay were higher in letrozole- and tamoxifen-treated CYP19A1 but the difference was not statistically significant (CYP19A1 control: none detectable; letrozole: 1.8 ± 1.1; tamoxifen: 1.4 ± 0.5) (Figure 1E). Representative whole mounts demonstrate that both letrozole and tamoxifen reduced overall ductal density but HANS remain demonstrable on tamoxifen treatment (Figure 1F). Representative sections illustrating mammary epithelial cells with nuclear-localized Ki67 (Figure 1G) and TUNEL staining (Figure 1H) are shown.

Esr1 and CYP19A1 transgenic mice differentially activate ER expression, pSTAT3 and pSTAT5 upon treatment with tamoxifen or letrozole

Percentages of mammary epithelial cells demonstrating nuclear-localized ER were measured before and after treatment as increased ER following treatment has been associated with tamoxifen resistance (46). The percentage of ER expressing mammary epithelial cells was significantly higher with tamoxifen treatment in Esr1 overexpressing mice (control: 6.8 ± 1.3 versus tamoxifen: 15.0 ± 3.4, P < 0.05, Mann–Whitney, unpaired, two-tailed) (Figure 2A). Although ER expression levels were unchanged by letrozole in Esr1 overexpressing mice (6.9 ± 1.4), they were significantly lower in letrozole-treated CYP19A1 overexpressing mice (control: 16.0 ± 3.2 versus letrozole: 5.0 ± 2.3, P < 0.05, Mann–Whitney, unpaired, two-tailed) (Figure 2A). Percentages of mammary epithelial cells demonstrating nuclear-localized PGR were measured because tamoxifen is reported to increase PGR expression levels in human breast cancer (47). Modest non-statistically significant increases were seen in both models (Esr1 control: 5.1 ± 2.0, letrozole: 3.5 ± 0.9, tamoxifen: 11.0 ± 5.5; CYP19A1 control: 10.0 ± 2.5, letrozole: 4.3 ± 2.3, tamoxifen: 14.0 ± 4.0) (Figure 2B). Percentages of mammary epithelial cells expressing cyclin D1, cyclin E, pSTAT3 and pSTAT5 were measured because overexpression of these proteins has been associated with tamoxifen resistance (11–14). Cyclin D1 expression was significantly lower in CYP19A1 overexpressing mice on letrozole treatment (control: 67 ± 4.3 versus letrozole: 36 ± 4.5, P < 0.05, Mann–Whitney, unpaired, two-tailed) but did not change significantly from control in any other treatment group (Esr1 control: 48 ± 10.2, letrozole: 69 ± 6.0, tamoxifen: 41 ± 10.9; CYP19A1 tamoxifen: 63 ± 10.5) (Figure 2C). No statistically significant differences were observed in percentages of cells with nuclear-localized cyclin E on either treatment (Esr1 control: 27 ± 5.6, letrozole: 36 ± 11.2, tamoxifen: 39 ± 7.7; CYP19A1 control: 18.2 ± 6.6, letrozole: 18 ± 4.2, tamoxifen: 31 ± 1.3) (Figure 2D). In Esr1 mice, the percentages of mammary epithelial cells demonstrating nuclear-localized pSTAT3 were significantly higher following letrozole treatment (control: 48 ± 8.1, letrozole: 79 ± 5.6, P < 0.05, Mann–Whitney, unpaired, two-tailed) but this was not seen with tamoxifen treatment in Esr1 mice (38 ± 10.3) or in CYP19A1 mice on either treatment (control: 62 ± 4.9, letrozole: 59 ± 11.8, tamoxifen: 50 ± 7.2) (Figure 2E). Differential responses following letrozole treatment were also found for the percentage of mammary epithelial cells with nuclear-localized pSTAT5 between the two models (Figure 2F). Although pSTAT5 percentages were significantly higher following letrozole treatment in Esr1 mice (control: 37 ± 13.3, letrozole: 84 ± 1.0, both P < 0.05, Mann–Whitney, unpaired, two-tailed), they were significantly lower in letrozole-treated CYP19A1 mice (control: 65 ± 0.4, letrozole: 23 ± 8.3, P < 0.05, Mann–Whitney, unpaired, two-tailed). They were not significantly altered, compared with respective controls, in tamoxifen-treated Esr1 (17 ± 5.5) or CYP19A1 (68 ± 17.5) mice.
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IRF7 and other immune associated genes are linked to tamoxifen resistance

Transcriptome analysis by RNA-seq was conducted to determine if significant differences in mammary gland gene expression could be identified in tamoxifen-resistant Ersl overexpressing and Brca1 KO mice compared with tamoxifen-sensitive CYP19A1 overexpressing mice prior to treatment. Totals of 837, 291 and 359 genes were defined as DEGs in the GEM (Brca1 KO: 575 upregulated, 262 downregulated; Ersl: 221 upregulated, 70 downregulated; CYP19A1: 114 upregulated, 245 downregulated) compared with the corresponding WT transcriptome (Figure 3A). Gene ontology analyses of the identified DEGs revealed unique characteristics of the transcriptomes of the two tamoxifen-resistant GEM (Figure 3B, Supplementary Table I, available at Carcinogenesis Online). Genes

Fig. 1. Comparison of changes in HAN prevalence and number, DH prevalence and proliferative and apoptotic indices in Ersl and CYP19A1 overexpressing mice with tamoxifen and letrozole treatment. (A) Bar graphs comparing HAN prevalence in control untreated, letrozole-treated and tamoxifen-treated Ersl and CYP19A1 overexpressing mice. Ersl control: 67%, n = 7; letrozole: none detected, n = 7; tamoxifen: 36%, n = 9; CYP19A1 control: 60%, n = 10; letrozole: none detected, n = 6; tamoxifen: 14%, n = 11. *P < 0.05, Fisher's exact. (B) Bar graphs comparing number of HANS/gland in control untreated, letrozole-treated and tamoxifen-treated Ersl and CYP19A1 overexpressing mice. Ersl control: 2.0 ± 0.9, n = 7; letrozole: none detected, n = 6; tamoxifen: 0.1 ± 0.1, n = 11. *P < 0.05, t-test, unpaired, one-tailed. Mean and standard error of the mean indicated. (C) Bar graphs comparing DH prevalence in control untreated, letrozole-treated and tamoxifen-treated Ersl and CYP19A1 overexpressing mice. Ersl control: 40%, n = 7; letrozole: none detected, n = 7; tamoxifen: 12%, n = 9; CYP19A1 control: 31%, n = 10; letrozole: none detected, n = 6; tamoxifen: 9%, n = 11. *P < 0.05, Fisher's exact. (D) Bar graphs comparing proliferative indices in control untreated, letrozole-treated and tamoxifen-treated Ersl and CYP19A1 overexpressing mice. Ersl control: 10.1 ± 4.5, n = 5; letrozole: 2.3 ± 1.1, n = 5; tamoxifen: 5.7 ± 0.9, n = 7. *P < 0.05, Mann–Whitney, unpaired, one-tailed. (E) Bar graphs comparing apoptotic indices in control untreated, letrozole-treated and tamoxifen-treated Ersl and CYP19A1 overexpressing mice. Ersl control: 0.5 ± 0.5, n = 4; letrozole: 0.5 ± 0.3, n = 4; tamoxifen: 0.8 ± 0.4, n = 5; CYP19A1 control: none detectable, n = 3; letrozole: 1.8 ± 1.1, n = 5; tamoxifen: 1.4 ± 0.5, n = 5. (F) Representative images of mammary gland whole mounts from control untreated, letrozole-treated and tamoxifen-treated Ersl and CYP19A1 overexpressing mice. Arrows indicate HANs. Size bar = 1 mm. (G) Representative images of Ki67 IHC on sections of mammary gland tissue from control untreated, letrozole-treated and tamoxifen-treated Ersl and CYP19A1 overexpressing mice. Arrows indicate representative mammary epithelial cells with nuclear-localized staining for Ki67. Images taken at ×40. Size bar = 20 µm. (H) Representative images of TUNEL assay on sections of mammary gland tissue from control untreated, letrozole-treated and tamoxifen-treated Ersl and CYP19A1 overexpressing mice. Arrows indicate representative TUNEL-labeled mammary epithelial cells. Images were taken at ×40. Size bar = 20 µm.
Fig. 2. Comparison of changes in percentages of mammary epithelial cells demonstrating nuclear-localized expression of ER, PGR, cyclin D1, cyclin E, phosphorylated STAT3 and phosphorylated STAT5 in Esr1 and CYP19A1 overexpressing mice with tamoxifen and letrozole treatment. (A) Bar graphs and representative histological images of IHC comparing percentage of mammary epithelial cells demonstrating nuclear-localized ER expression from control untreated, letrozole-treated and tamoxifen-treated Esr1 and CYP19A1 overexpressing mice. Esr1 control: 6.8 ± 1.3, n = 6; letrozole: 6.9 ± 1.4, n = 7; tamoxifen: 15.0 ± 3.4, n = 8; CYP19A1 control: 16.0 ± 3.2, n = 7; letrozole: 5.0 ± 2.3, n = 5; tamoxifen: 11.0 ± 1.5, n = 4. (B) Bar graphs and representative histological images of IHC comparing percentage of mammary epithelial cells demonstrating nuclear-localized PGR expression from control untreated, letrozole-treated and tamoxifen-treated Esr1 and CYP19A1 overexpressing mice. Esr1 control: 5.5 ± 2.0, n = 6; letrozole: 3.5 ± 0.9, n = 7; tamoxifen: 11.0 ± 3.5, n = 8; CYP19A1 control: 10.0 ± 2.5, n = 8; letrozole: 4.3 ± 2.3, n = 5; tamoxifen: 14.0 ± 1.4, n = 7. (C) Bar graphs and representative histological images of IHC comparing percentage of mammary epithelial cells demonstrating nuclear-localized cyclin D1 expression from control untreated, letrozole-treated and tamoxifen-treated Esr1 and CYP19A1 overexpressing mice. Esr1 control: 48 ± 10.2, n = 5; letrozole: 69 ± 6.0, n = 8; tamoxifen: 41 ± 10.9, n = 8; CYP19A1 control: 67 ± 4.3, n = 13; letrozole: 36 ± 4.5, n = 11; tamoxifen: 63 ± 10.5, n = 7. (D) Bar graphs and representative histological images of IHC comparing percentage of mammary epithelial cells demonstrating nuclear-localized cyclin E expression from control untreated, letrozole-treated and tamoxifen-treated Esr1 and CYP19A1 overexpressing mice. Esr1 control: 27 ± 5.6, n = 5; letrozole: 36 ± 11.2, n = 5; tamoxifen: 39 ± 7.7, n = 4; CYP19A1 control: 18.2 ± 6.6, n = 6; letrozole: 18 ± 4.2, n = 6; tamoxifen: 31 ± 1.3, n = 4. (E) Bar graphs and representative histological images of IHC comparing percentage of mammary epithelial cells demonstrating nuclear-localized pSTAT3 expression from control untreated, letrozole-treated and tamoxifen-treated Esr1 and CYP19A1 overexpressing mice. Esr1 control: 48 ± 8.1, n = 6; letrozole: 79 ± 5.6, n = 8; tamoxifen: 38 ± 10.3, n = 8; CYP19A1 control: 62 ± 4.9, n = 13; letrozole: 59 ± 11.8, n = 7; tamoxifen: 50 ± 7.2, n = 12. (F) Bar graphs and representative histological images of IHC comparing percentage of mammary epithelial cells demonstrating nuclear-localized pSTAT5 expression from control untreated, letrozole-treated and tamoxifen-treated Esr1 and CYP19A1 overexpressing mice. Esr1 control: 37 ± 13.3, n = 3; letrozole: 84 ± 1.0, n = 5; tamoxifen: 17 ± 5.5, n = 5; CYP19A1 control: 65 ± 0.4, n = 7; letrozole: 23 ± 8.3, n = 6; tamoxifen: 68 ± 17.5, n = 2. *P < 0.05 Mann–Whitney, unpaired, two-tailed. Mean and standard error of the mean indicated. Arrows indicate representative stained mammary epithelial cells. Images taken at ×40. Size bar = 20 μm.

associated with ‘immune system process’ and ‘response to stimulus’ were those most notably upregulated in tamoxifen-resistant Esr1 overexpressing and Bref1 KO mice, whereas genes related to ‘cellular aromatic compound metabolic process’ were specifically upregulated in CYP19A1 overexpressing mice. Non-overlapping genes related to immune response were upregulated in each tamoxifen-resistant model compared with WT mice, whereas cell adhesion genes were upregulated specific to CYP19A1 overexpression mice compared with WT mice (Supplementary Figure 1, available at Carcinogenesis Online). Sixty-eight genes were identified as being uniquely upregulated in the two tamoxifen-resistant mouse models compared with the tamoxifen-sensitive model (Figure 3C). The 28 genes with the most significant differences in expression patterns are illustrated in Figure 3D. For further insight into functions of the 68 genes, gene ontology and network analyses were performed via Cytoscape. The majority of the 68 uniquely upregulated genes were definitively associated with immune response including IFN-alpha/beta/gamma pathways (Iif1, Iif3, Gbp9, I30012016Rik and H2-Ab1), antigen processing (Cis, H2-Ab1, H2-Q2, H2-Q7 and H2-Q8) and inflammatory response (Alox5 and Serpin1) (Figure 3E, Supplementary Table II, available at Carcinogenesis Online). To work toward identifying a key transcription factor regulating these genes, a molecular network using GeneMANIA was recognized and a core network extracted from it using MCODE (Figure 3F). Significantly, Irf7, a key transcriptional regulator of IFN-dependent immune responses, was identified. This transcription factor activates target genes by binding to a consensus DNA motif (AAAnGAAA) in their promoters. To test whether the 68 genes were controlled by IRF7 or transcription factors, the significantly overrepresented TFBSs on the promoters of these 68 genes were examined (Figure 3G). Intriguingly, the IRF-binding site was the most significant motif among the known 130 TFBSs. Two loci
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encoding novel transcripts showing upregulation specific to the tamoxifen-resistant mouse models were identified (Figure 4A). At least four transcripts with high homology to human immunoglobulin lambda-like polypeptide (IGLL) 1, IGLL3P, IGLL5, rabbit IGLL1 and cow IGLL1 were expressed from the XLOC_010544 locus. Transcripts a, c and e are highly likely to be protein-coding genes (Figure 4B) and transcripts b, c and e were specifically upregulated in the tamoxifen-resistant Esr1 overexpressing and Brca1 KO mice.
Expression levels of KO GEM models. (A) Normalized read coverage across the XLOC_010544 and XLOC_00615 loci viewed through the integrative genomics viewer illustrating relative expression levels in WT, CYP19A1KO and Breca1 KO mice. The potential IRF-binding sites and exon structures of the assembled transcripts (a, b, c, d, e) are indicated. Sequence homology of XLOC_010544 with human IGLL5, human IGLL3P, human IGLL1, rabbit IGLL1 and cow IGLL1 shown. (B) Bar graphs indicating relative probability of coding potential of the novel transcripts assessed using the Coding-Potential Assessment Tool. Transcripts showing coding probability below 0.44 were regarded as non-coding RNAs. *Indicates the three transcripts (a, c, e) with statistically significant scores for coding potential. (C) Bar graphs illustrating relative fragments per kilobase of transcript per 68 million mapped reads of the five different assembled transcripts from the XLOC_010544 and XLOC_00615 loci (a, b, c, d, e) in WT (gray), CYP19A1KO (black-outlined gray), Esr1 (white) and Breca1 KO (black) mice.

**Discussion**

The greater efficacy of letrozole compared with tamoxifen for resolution of mammary preneoplasia shown here parallels results reported in women where aromatase inhibitors have shown a higher response rate (15, 16, 19). In the tamoxifen-resistant Esr1 mice, tamoxifen was unable to resolve the larger HANs that represent a later stage in disease progression although it was effective in reducing prevalence of the smaller DHs that occur at an earlier stage in disease progression, compatible with previous studies in this model that showed that tamoxifen could reduce ductal abnormalities if administered at 4 months of age (1.5). Tamoxifen resistance in this model is correlated with disease progression suggesting that the timing of a preventive intervention may be a key factor in its effectiveness. Timing of preventive interventions in relationship to disease progression is an issue to consider for breast cancer prevention in women as well. Significantly, the aromatase inhibitor letrozole was effective at reducing both early and late lesions. If aromatase inhibitors are more uniformly effective at both early and later stages of disease progression, this could be a factor favoring their selection. At the same time, this is complicated by the fact that only tamoxifen has an acceptable safety profile in premenopausal women, the same population that may be most likely to exhibit early disease.

The molecular effects of the anti-hormonal agents in the GEM models studied here showed significant parallels with reports from human populations. Decreased proliferation is linked to an effective response to anti-hormonal agents in women (48). Letrozole, compared with tamoxifen, mediated a more dramatic reduction of levels of mammary epithelial cell proliferation and may have contributed to the more profound impact on preneoplasia. Esr1 overexpressing mice showed a statistically significant increase in ER levels, a finding associated with acquisition of tamoxifen resistance *in vitro* (46). The higher, but variable and not statistically significant, apoptotic index found with tamoxifen treatment in the CYP19A1 overexpressing mice compared with Esr1 overexpressing mice suggests but does not prove that higher levels of apoptosis with tamoxifen in the CYP19A1 overexpressing
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mice contributed toward its greater effectiveness. Induction of apoptosis is linked to tamoxifen response (49) and activation of anti-apoptotic cell survival mechanisms is associated with tamoxifen resistance (12).

A reduction in cyclin D1 levels was correlated with a positive response to letrozole but only in CYP19A1 overexpressing and Brca1 KO mice. It is possible that molecular responses on therapy correlating with disease response may depend upon the initiating event. This could be a reason for the occasionally diverse responses reported in human studies and the difficulty in identifying robust and reproducible molecular markers of response in non-homogeneous populations. The difference in the pSTAT5 response was even more dramatic between the two models. pSTAT5 has two functions in mammary epithelial cells, differentiation and proliferation (50). In Esr1 overexpressing mice, loss of STAT5 impairs mammary epithelial cell differentiation during puberty (2). It is theoretically possible that increased differentiation mediated by pSTAT5 contributed to the positive response to letrozole in the Esr1 mice as decreased proliferation accompanies increased differentiation in the mammary gland. It is conceivable that at the same time the drop in pSTAT5 in the CYP19A1 mice was a more direct factor in decreasing mammary epithelial cell differentiation rates. Such a differential impact of the same drug in different models of estrogen-pathway-mediated disease illustrates a potential reason for the difficulty in establishing predictive markers that work across different breast cancer risk populations.

Fig. 5. Localization of IRF7, STAT1, CD45, CD3 and F4 80 staining in cells labeled by IHC in mammary gland and adenocarcinoma tissue. (A) Representative histological images of IHC for nuclear-localized IRF7 (large panels) and STAT1 (insets) in mammary epithelial cells from control untreated WT, Esr1 and CYP19A1 overexpressing and Brca1 KO mice. (B) Representative histological images of IHC for membrane-localized CD45 staining in mammary gland tissue from control untreated WT, Esr1 and CYP19A1 overexpressing and Brca1 KO mice. (C) Representative histological images of IHC for predominantly membrane-localized CD3 in mammary gland tissue from control untreated WT, Esr1 and CYP19A1 overexpressing and Brca1 KO mice. (D) Representative histological images of IHC for F4 80 in mammary gland tissue from control untreated WT, Esr1 and CYP19A1 overexpressing and Brca1 KO mice. (E) Representative histological image of hematoxylin and eosin staining of mammary adenocarcinoma tissue from a Brca1 KO mouse. (F) Representative histological image of IHC for CD45 staining in mammary gland tissue from a Brca1 KO mouse. (G) Representative histological image of IHC for CD3 staining in mammary gland tissue from a Brca1 KO mouse. (H) Representative histological image of IHC for F4 80 in mammary adenocarcinoma tissue from a Brca1 KO mouse. Arrows indicate representative stained mammary epithelial cells (A), leukocytes (B and F), T cells (C and G), macrophages (D and H). Images taken at ×40. Size bar = 20 µm.
Fig. 6. IRF7 knockdown results in decreased expression of predicted targets PARP14, IRGM1 and GBP7. (A) Representative western blot analysis of IRF7 and actin expression in MCF-7 cells under basal untreated (-), scrambled negative control siRNA (C) and IRF7 siRNA (IRF7) conditions 1 and 3 days following transfection. Size markers are indicated on the gel at left. Arrows at right indicate bands running at the appropriate size for IRF7 (54kDa) and actin (42kDa). Images shown illustrate results for both proteins on the same blot. Images cropped from the original scanned films. (B) Bar graphs showing relative expression levels of IRF7 normalized to actin (mean ± SEM) for basal (-), scrambled negative control siRNA (C) and IRF7 siRNA (IRF7) conditions 3 days after transfection. Relative expression levels under basal (-) conditions set to 1. N = 3 independently performed transfections followed by western blot analyses. (C) Images of ethidium bromide stained agarose gels showing intensity of bands following reverse transcriptase–PCR for primer sets #1 (130 bp) and #2 (100 bp) for IRF7 at 1:1 and 1:5 dilutions, ACTB (100 bp) at 1:1 dilution and GAPDH (100 bp) at 1:5 dilution. Molecular weight markers (100–2000 bp, exACTGene, low range plus DNA ladder, Fisher Scientific International, Waltham, MA) illustrated at left. Arrows and arrowheads at right of gels indicate position of bands of expected size. *indicates position of non-specific bands. Images shown cropped from original digital images of the gels. (D) Bar graphs showing relative expression levels of PARP14 normalized to actin (mean ± SEM) for basal (-), scrambled negative control siRNA (C) and IRF7 siRNA (IRF7) conditions 3 days after transfection and representative western blots. Relative expression levels under basal (-) conditions set to 1. N = 3 independently performed transfections followed by western blot analyses. Size of products indicated at right (original size markers on gels cropped off). Images show results for both proteins on the same blot, cropped from the original scanned films. (E) Bar graphs showing relative expression levels of IRGM1 normalized to actin (mean ± SEM) for basal (-), scrambled negative control siRNA (C) and IRF7 siRNA (IRF7) conditions 3 days after transfection and representative western blots. Relative expression levels under basal (-) conditions set to 1. N = 3 independently performed transfections followed by western blot analyses. Size of products indicated at right (original size markers on gels cropped off). Images show results for both proteins on the same blot, cropped from the original scanned films. (F) Bar graphs showing relative expression levels of GBP7 normalized to actin (mean ± SEM) for basal (-), scrambled negative control siRNA (C) and IRF7 siRNA (IRF7) conditions 3 days after transfection and representative western blots. Relative expression levels under basal (-) conditions set to 1. N = 3 independently performed transfections followed by western blot analyses. Size of products indicated at right (original size markers on gels cropped off). *indicates position of non-specific bands. Images show results for both proteins on the same blot, cropped from the original scanned films.
Transcriptome analysis demonstrated that the two different interventions in estrogen signaling, increased ER and increased aromatase, have significantly different effects on the mammary gland transcriptome. Whereas ‘response to stimulus’ and ‘immune system process’ were the two most significant terms associated with upregulated DEGs in Esr1 mice, ‘cellular aromatic compound metabolic process’ and ‘folate acid transport’ were the two most significant terms in Cyp19a1 mice. These different impacts on transcription may underlie the differences in anti-hormonal response documented in this study. Notably, ‘immune system process’ and ‘response to stimulus’ were also the two most significant terms associated with upregulated DEGs in tamoxifen-resistant Brca1 KO mice (6). This raises the possibility that immune gene-related mechanisms may mediate tamoxifen resistance. Upregulation of immune gene expression in epithelial cells is associated with increased cell survival and resistance to DNA damage and cispaltin (27,28), the same may be occurring here in response to tamoxifen. Irf7 was identified as the most highly upregulated Irf family member in both tamoxifen-resistant models and this is a gene reported to be upregulated in human breast cancer as well (29).

Nuclear-localized Irf7 was present in mammary epithelial cells of the tamoxifen-resistant models and regulation of specific gene products predicted by the bioinformatics analyses was experimentally confirmed in human breast cancer cells. Both epithelial and stromal compartments can contribute to disease progression and therapy resistance (23–25). Here, whole mammary tissue was studied and the alterations in gene transcription may have been derived from either or both compartments. Infiltration of inflammatory cells adjacent to mammary preneoplasia may be the source for IFNs triggering activation of IRFs and downstream genes. Specification of two loci containing novel immune-related transcripts led to the identification of the mouse homologue for human IGLL and a second transcript with high coding potential but whose function remains unknown revealing the power of RNA-seq in providing new and unanticipated information. Significantly, both transcripts contain TFBSs for IRF, compatible with their upregulation by Irf7.

In summary, this study showed differential responses to tamoxifen and letrozole with two different lesions in the estrogen–ER pathway found that letrozole can be effective in the face of tamoxifen resistance and demonstrated as association between altered immune-related gene expression and tamoxifen resistance. The results would support development of translational studies using human tissue to further examine a possible association between alterations in immune-related gene expression and the probability of tamoxifen resistance. Additional work will be required to establish the relative contributions of the stroma and epithelial compartments to the altered gene signatures and the possible role of stromal–epithelial crosstalk in mediating these changes. Understanding genetic alterations during cancer progression could be facilitated by establishing the exact genome-wide Irf7 occupancy using chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) and subsequent regulation of genetic circuits in the different cell populations.

Supplementary material

Supplementary Tables I and II and Figures 1 and 2 can be found at http://cancer.oxfordjournals.org/

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References


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