Differences in the Transcriptional Response to Fulvestrant and Estrogen Deprivation in ER-Positive Breast Cancer

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Abstract

Purpose: Endocrine therapies include aromatase inhibitors and the selective estrogen receptor (ER) downregulator fulvestrant. This study aimed to determine whether the reported efficacy of fulvestrant over anastrozole, and high- over low-dose fulvestrant, reflect distinct transcriptional responses.

Experimental Design: Global gene expression profiles from ERα-positive breast carcinomas before and during presurgical treatment with fulvestrant (n = 22) or anastrozole (n = 81), and corresponding in vitro models, were compared. Transcripts responding differently to fulvestrant and estrogen deprivation were identified and integrated using Gene Ontology, pathway and network analyses to evaluate their potential significance.

Results: The overall transcriptional response to fulvestrant and estrogen deprivation was correlated (r = 0.61 in presurgical studies, r = 0.87 in vitro), involving downregulation of estrogen-regulated and proliferation-associated genes. The transcriptional response to fulvestrant was of greater magnitude than estrogen deprivation (slope = 0.62 in presurgical studies, slope = 0.63 in vitro). Comparative analyses identified 28 genes and 40 Gene Ontology categories affected differentially by fulvestrant. Seventeen fulvestrant-specific genes, including CAV1/2, SNAI2, and NRPI, associated with ERα, androgen receptor (AR), and TP53, in a network regulating cell cycle, death, survival, and tumor morphology. Eighteen genes responding differently to fulvestrant specifically predicted antiproliferative response to fulvestrant, but not anastrozole. Transcriptional effects of low-dose fulvestrant correlated with high-dose treatment, but were of lower magnitude (ratio = 0.29).

Conclusions: The transcriptional response to fulvestrant has much in common with estrogen deprivation, but is stronger with distinctions potentially attributable to arrest of estrogen-independent ERα activity and involvement of AR signaling. Genes responding differently to fulvestrant may have predictive utility. These data are consistent with the clinical efficacy of fulvestrant versus anastrozole and higher dosing regimens. Clin Cancer Res; 1–12. ©2014 AACR.

Introduction

Endocrine therapies abrogate estrogenic signaling through distinct mechanisms, impeding estrogen synthesis or transcriptional activity of estrogen receptor alpha (ERα).

Aromatase inhibitors, for example, anastrozole, cause profound postmenopausal estrogen suppression and are used as first-line neoadjuvant, adjuvant, and metastatic therapies. Selective estrogen receptor modulators (SERM), for example, tamoxifen, exert partial agonist activity. In contrast, the selective ER downregulator (SERD) fulvestrant (Faslodex, AstraZeneca) is a pure antiestrogen, inhibiting receptor dimerization, nuclear uptake, estrogen-response element binding, and accelerating ERα degradation (1–5).

Fulvestrant is licensed for postmenopausal progression/relapse on first-line endocrine therapy (6–8). Low-dose fulvestrant (250 mg/28 days) provides comparable disease outcome to anastrozole following first-line tamoxifen in metastatic disease (6–8), with utility after progression on aromatase inhibitors (9–11). As first-line therapy in metastatic or locally advanced disease, low-dose fulvestrant provides comparable disease outcome to tamoxifen (12). High-dose treatment (500 mg on day 0, 14, 28, monthly thereafter) further improved progression-free (13) and overall survival.

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Translational Relevance

Aromatase inhibitors are first-line postmenopausal agents for estrogen receptor alpha (ERα)-positive breast cancer. However, there is considerable response heterogeneity and women frequently relapse. Estrogen deprivation does not completely arrest ERα activity, and transactivation of the unliganded receptor may continue through cross-talk with growth factor pathways. In contrast with aromatase inhibitors, the selective ER down-regulator fulvestrant also abrogates ligand-independent ERα activity. The benefit of fulvestrant as an alternative, combination, or sequential therapy to aromatase inhibitor has been reported, but molecular mechanisms underpinning its relative efficacy remain unclear and biomarkers for patient selection are lacking. This study demonstrates, for the first time, that the overall transcriptional response to fulvestrant is of greater magnitude than estrogen deprivation, consistent with its clinical efficacy and more complete blockade of estrogenic signaling. Using a robust integrative approach, we identify a subset of genes differentially affected by fulvestrant that comprises distinct biologic networks, correlates with antiproliferative response, and has potential utility as predictive biomarkers for fulvestrant.

Materials and Methods

Presurgical study of fulvestrant

Pre- and on-treatment (4-week) core biopsies stored at −20°C in RNA-later (Qiagen) were available from NEWEST (ClinicalTrials.gov-NCT00093002; ref. 17; Supplementary Fig. S1). This phase II study recruited postmenopausal women with untreated, potentially operable, locally advanced, ERα-positive, primary invasive cancer ≥2 cm. No data were available for HER2 status. Randomization was to low- (250 mg/28 days) or high-dose (500 mg on days 0, 14, 28, monthly thereafter) fulvestrant. The on-treatment biopsy was taken before the day 28 dose of fulvestrant in both arms of the study. RNA was extracted with RNAeasy, assessed using an Agilent Bioanalyser (Santa Clara) and rejected if RNA integrity number was <5. Following exclusions, 22 high-dose and 16 low-dose pre-/on-treatment pairs were available (Supplementary Fig. S1).

Presurgical study of anastrozole

Pre- and on-treatment (2- and 16-week) core biopsies were available from postmenopausal women receiving anastrozole monotherapy (1 mg/day) within a randomized phase II neoadjuvant trial of anastrozole alone or with gefitinib in early disease (ClinicalTrials.gov-NCT00255463; ref. 28). This subgroup constitutes the Functional Aromatase Inhibitor Molecular Study (FAIMoS; ref. 29; Supplementary Fig. S1B and S1C). Following exclusions, 81 two-week and 18 sixteen-week pairs were available (Supplementary Fig. S1). Written informed consent was obtained from each subject and investigations performed after approval by a local Institutional Review Board.
**In vitro** modeling of fulvestrant or estrogen deprivation

MCF7 cells (ATCC) were cultured in phenol red-free RPMI-1640, 10% FBS (Gibco Life Technologies), and 1 nmol/L 17β-estradiol (E2). Cells were stripped of steroids for 48 hours in phenol red-free RPMI with 10% dextran-coated charcoal-stripped FBS (DCC). Cells were seeded into 6-well plates at a density of 3 x 10^5 cells/well for 24 hours. Monolayers were: (i) harvested at this stage, that is, following 72 hours of estrogen deprivation (modeling aromatase inhibitor), (ii) treated for 48 hours with 0.1 nmol/L E2 in DCC (modeling baseline), or (iii) treated for 48 hours with 10 nmol/L fulvestrant and 0.1 nmol/L E2 (modeling fulvestrant). Experiments were conducted in triplicate and RNA extracted using RNeasy (Qiagen).

Microarray-based global gene expression profiling

RNA was quantified, amplified, labeled, and hybridized onto Expression BeadChips (Illumina). Samples were processed with the following BeadChips: (i) FAIMoS - HumanWG-6v2, (ii) NEWEST - HumanWG-6v3, (iii) bridging study of ten high-dose pairs from NEWEST and ten two-week pairs from FAIMoS - HumanHT-12v4, and (iv) **in vitro** samples - HumanHT-12v4.

**Global gene expression analysis**

Raw expression data were extracted with BeadStudio, transformed by variance-stabilizing transformation and normalized using Robust Spline Normalization in the Lumi package in Bioconductor. Probes were excluded if they were not present in any samples (detection P>1%). Microarray data are publicly available (29–32). Expression data and annotation files (HumanHT-12_V4_0_R2_15002873_B, HumanWG-6_V3_0_R3_11282955_A, and HumanWG-6_V2_0_R4_11223189_A) were imported into Partek Genomics Suite (PGS, 6.6.6.12.0531, Partek Incorporated).

Class comparison of pre- and on-treatment clinical samples used two-way ANOVA. Treatment status (i.e., pre- or on-treatment) was considered a categorical variable with fixed effect (as assignment represents all conditions of interest). Pre- and on-treatment samples were paired according to their patient identifier, which was considered.

![Figure 1](image-url). A, summary of estrogen-dependent ERα signaling (yellow) and the potential independent activities of estrogen (blue) and ERα (red). B, comparison of the impact of aromatase inhibitors and SERDs on classical and nonclassical estrogenic signaling, illustrating activities targeted by both agents (yellow) and those affected specifically by aromatase inhibitors (blue) or fulvestrant (red). Reduced activity is indicated by downward arrows (solid black) and no change is indicated by horizontal arrows (black outline).
a random effect variable which encompassed inter-patient variability (given that patients represent a random sample of all possible patients). Transcripts differentially expressed between fulvestrant-treated, estrogen-deprived, and control conditions in vitro were identified by ANOVA. FDR of 5% was used to correct for multiple testing.

**Analytical strategy**

Matched Illumina probe identifiers were used to enable valid comparisons between BeadChips. All detected probes from HumanWG-6v3 (n = 22550) were used to evaluate fulvestrant dosing regimens. Comparisons between NEWEST and FAIMoS used detected probes common to HumanWG-6v3 and HumanWG-6v2 (n = 15051), followed by an unpaired t test of treatment-induced alterations. Detected probes common to HumanHT-12v4, HumanWG-6v3, and HumanWG-6v2 (n = 11122) were assessed in vitro. Biologic interpretation involved: (i) identification of functional groupings from the Gene Ontology database, by Gene Ontology ANOVA in PGS and (ii) network analyses with Ingenuity Pathway Analysis (IPA; Ingenuity Systems).

**Technical validation of microarray findings for selected genes**

Expression of two selected genes, CAV1 and SNAI2, was assessed by qRT-PCR of the same RNA preparations used for expression profiling of high-dose fulvestrant (n = 20) and 2-week anastrozole (n = 31)-treated patients. TaqMan assays (Applied Biosystems) were used to quantify CAV1 (Hs0971716_m1) and SNAI2 (Hs0950344_m1), which were normalized to FKBPI5 (Hs03914800_m1) and PUM1 (Hs09827755_m1).

**ESR1 knockdown in MCF7 cells**

MCF7 cells were seeded into DCC at a density of 7 × 10^4 cells/well in 12-well plates. After 24 hours, monolayers were transfected with 50 nmol/L of siRNA targeting ESR1 (ON-TARGETplus 003401, Dharmacon, ThermoFisher), or non-targeting siRNA using DharmaFECT 3 reagent. Media (0.1 nmol/L E2 in DCC) were replenished the following day and cells were cultured for 24 hours. TaqMan assays were used to quantify ESR1 (Hs01046818_m1) and selected genes: BRI3 (Hs0854645_g1), CAV1, CAV2 (Hs0184597_m1), CCDC34 (Hs0293234_m1), CYP26B1 (Hs01011223_m1), C9orf140 (Hs0746788_s1), FNTA (Hs0357739_m1), GTSE1 (Hs0212681_m1), HMGN4 (Hs01549435_m1), LRP8 (Hs0182998_m1), NRPI (Hs0826128_m1), NSUN2 (Hs0214829_m1), RBMS1 (Hs0377856_m1), RECL4 (Hs01548660_g1), SEPI1 (Hs01032845_m1), SLC7A5 (Hs0185826_m1), SNAI2, and STRA13 (Hs0414534_m1), which were normalized to FKBPI5.

**Results**

**Transcriptional response to high- and low-dose fulvestrant**

The overall transcriptional response to low-dose fulvestrant was significantly correlated with that to high-dose fulvestrant (Pearson r = 0.36, P < 0.0001), albeit of lesser magnitude (slope = 0.29; Fig. 2A; Deming linear regression), supporting a quantitative difference between the dosing schedules. None of the alterations in gene expression induced by low-dose treatment were statistically significant after multiple testing corrections (FDR<0.05). However, downregulation of individual proliferation-associated (e.g., AURKA) or estrogen-regulated (e.g., PGR, PDZK1, and GREB1) genes reached significance (uncorrected P<0.05; Supplementary Table S1). In contrast, 2,210 transcripts were significantly affected (977 upregulated and 1,233 downregulated, FDR<0.05) in the high-dose cohort. Further comparative analyses were undertaken with only high-dose treated patients to avoid the potential for false negativity by inclusion of those receiving low-dose fulvestrant.

**Similarities in the transcriptional response to fulvestrant and estrogen deprivation**

The overall transcriptional response to anastrozole and high-dose fulvestrant in presurgical studies was significantly correlated (Pearson r = 0.61, P < 0.0001; Fig. 2B and C), as were those of estrogen deprivation and fulvestrant in vitro (Pearson r = 0.87, P < 0.0001; Fig. 2D). In both settings, ERGs (e.g., PDZK1, PGR, GREB1, and TFF1) were significantly downregulated by estrogen deprivation and fulvestrant; full listings are provided in Supplementary Tables S2–S4). Proliferation-associated genes were also downregulated by estrogen deprivation (e.g., TOP2A, CDC5, CDC20, CCNB2, AURKA, and E2F2) and fulvestrant (e.g., TOP2A, CCNA2, CCNB2, CCND1, CDC5, CDC7, CDC8, CDC2, CDC20, CDC25C, AURKA, and POLE; detailed in Supplementary Tables S2–S4). Proliferation-associated Gene Ontology sets, pathways, and networks were prominent in transcripts affected by both treatments (Supplementary Tables S5–S8). Ki-67 staining was comparably reduced in samples from patients receiving anastrozole (geometric mean of post-/pretreatment expression = 24.8% after 2 weeks, n = 69) and fulvestrant (19.6%, following high-dose treatment, n = 22).

**Differences in the transcriptional response to fulvestrant and estrogen deprivation**

The overall transcriptional response to high-dose fulvestrant was of greater magnitude than anastrozole in presurgical studies (slope = 0.62, Fig. 2B). This difference was supported by the bridging study (slope = 0.56, Fig. 2C), excluding batch separation as a potential explanation, and consistent with the greater impact of fulvestrant compared with estrogen deprivation in vitro (slope = 0.63, Fig. 2D). To determine whether any transcripts were affected differently by the agents, rigorous comparisons were undertaken following the scheme illustrated in Fig. 3A. Treatment-related transcripts were identified that met the following criteria: (i) expression changed significantly after treatment in clinical samples and corresponding in vitro models (FDR<0.05), (ii) expression was affected differently by the two agents in vitro (discovery set, FDR<0.05) and in presurgical studies...
(validation set, uncorrected $P < 0.05$), and (iii) changes in expression were consistent in clinical samples and corresponding in vitro models (direction of change and relative treatment effect). Transcript sets from Fig. 3A are detailed in Supplementary Table S2.

Fulvestrant-related transcripts ($n = 41$, 13 upregulated and 28 downregulated) and estrogen deprivation–related transcripts ($n = 18$, 3 upregulated and 15 downregulated) were then assessed for whether differences were treatment specific or quantitative (Fig. 3A). Of the 15 genes downregulated by estrogen deprivation, four ($KCNK6$, $KCNK15$, $RASGRP1$, and $TUBA3E$) were not similarly affected by fulvestrant, whereas for the 28 downregulated by fulvestrant, 17 were not similarly affected by estrogen deprivation (e.g., $GTSE1$, $LRP8$, $NSUN2$, and $STRA13$, $P = 0.005$ for the comparison of those not similarly downregulated, i.e., 4 vs. 17, McNemar test); 11 genes were downregulated significantly more by fulvestrant than by estrogen deprivation (e.g., $CCDC34$, $RECL4$, $SLC7A5$, and $SAPCD2$). Of the three genes significantly upregulated by estrogen deprivation, only one ($DCXR$) was not similarly affected by fulvestrant, whereas for the 13 upregulated by fulvestrant, 11 were not similarly affected by estrogen deprivation (e.g., $CAV1$, $CAV2$, $SNAI2$, and $NRPI$, $P = 0.004$ for the comparison of those not similarly upregulated); two transcripts (e.g., $ZMAT3$) were upregulated significantly more by fulvestrant than estrogen deprivation. Treatment-related and -specific alterations are summarized in Fig. 3C; i; detailed in Supplementary Table S9.

To assess whether transcript differences might be sufficient to influence distinct biologic processes, differentially affected Gene Ontology sets were identified in the same

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**Figure 2.** Scattergrams illustrating the overall transcriptional response to fulvestrant or estrogen deprivation: A, low-dose compared with high-dose fulvestrant. B, high-dose fulvestrant compared with 2 weeks of anastrozole. C, bridging study of high-dose fulvestrant compared with 2 weeks of anastrozole. D, MCF7 cells modeling fulvestrant treatment and estrogen deprivation.
Fulvestrant downregulated 32 Gene Ontology sets significantly more than estrogen deprivation. Common downregulated sets were predominantly proliferation related (e.g., DNA helicase activity, microtubule motor activity, and spindle assembly; Supplementary Table S10). Of the 14 Gene Ontology sets upregulated by estrogen deprivation, two were not similarly affected by fulvestrant, whereas for the 49 upregulated by fulvestrant, 37 were not similarly affected by estrogen deprivation (e.g., apoptotic signaling pathway, and negative regulation of: ERK1/2 cascades, TGFβ receptor signaling, canonical Wnt receptor signaling, and epithelial cell proliferation, \( P < 0.001 \) for the comparison of those not similarly upregulated). Some Gene Ontology sets specifically upregulated by fulvestrant pertained to fulvestrant-specific upregulated genes (e.g., caveolae assembly and \( \text{CAV1/CAV2} \)). Fulvestrant upregulated 12 Gene Ontology sets significantly more than estrogen deprivation (e.g., negative regulation of MAPK cascade). Treatment-related and -specific alterations in Gene Ontology

Figure 3. Summary of analytical strategy with (A) resultant transcripts and (B) Gene Ontology functional gene sets. Key: In vitro models (yellow), presurgical studies (grey), control conditions in vitro/pretreatment clinical samples (C, black), estrogen deprivation (ED, blue), fulvestrant (F, red), ED and F (green). Transcripts and Gene Ontology sets resulting from comparative analyses are detailed in Web Appendix 1. C, summary of (i) transcripts and (ii) Gene Ontology functional gene sets affected by estrogen deprivation (ED, blue), fulvestrant (F, red), or both (green), with treatment-specific and quantitative differences indicated by horizontal and vertical stripes, respectively.
Pathway and network analysis of genes differentially affected by fulvestrant

Canonical pathways associated with fulvestrant-specific genes (n = 28, 11 upregulated and 17 downregulated) included signaling through heterotrimeric G-proteins (β-/γ-subunits) and estrogen-mediated S phase entry (Table 1). One principal network (IPA score = 40) included 17 of 28 fulvestrant-specific genes with functions related to cell cycle, death, survival, and tumor morphology (Fig. 4). Six fulvestrant-specific upregulated genes were present, with CAV1/2, SNAI2, and NRP1 forming a core group associated directly and/or indirectly with the focal points of ERα, androgen receptor (AR), and TP53. Members of the MAPK/ERK family and the serine/threonine protein kinase (AKT) were prominent. Eleven fulvestrant-specific downregulated genes occupied the network periphery.

To determine whether this functional alliance of fulvestrant-specific genes was specifically induced by, and attributable to, congruent response to fulvestrant, their pretreatment expression was assessed. Fulvestrant-related genes correlated significantly with one another at baseline; upregulated genes were coexpressed, as were downregulated genes, and these two groups were already inversely correlated, implicating a preexisting regulatory system. Fulvestrant downregulated genes correlated directly, whereas those upregulated correlated inversely, with baseline AURKA and ESR1 (summarized in Supplementary Fig. S2 and detailed in Supplementary Table S11).

Treatment-related gene alterations and antiproliferative response

To determine whether baseline expression of treatment-related genes (n = 46) was associated with antiproliferative response, their pretreatment levels were correlated with change in AURKA expression. Twenty-six genes correlated with response to anastrozole or high-dose fulvestrant (uncorrected P < 0.05, summarized in Fig. 5A and detailed in Supplementary Table S12). Twenty-three fulvestrant-related genes correlated with response to high-dose fulvestrant, with five of 23 (C9orf140, POLD2, SAC3D1, ZMYND19, and STRA13) also doing so in the low-dose treated cohort (Supplementary Table S12c). Notably, 18 of 23 were not associated with response to anastrozole. Fulvestrant response was specifically associated with low pretreatment expression of two genes (SNAI2 and NRP1) found to be upregulated by fulvestrant, and high expression of 16 genes found to be downregulated by fulvestrant; of these C9orf140, LRP8, and CCDC34 had predictive significance independent of pretreatment AURKA expression (P < 0.05, Fig. 5B, i–iii).

Pretreatment expression of five genes had predictive significance for both agents and their correlation was

Table 1. Ingenuity-based biologic interpretation of genes differentially affected by fulvestrant in presurgical studies and in vitro models: Network functions and canonical pathways

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<td>40</td>
<td>17</td>
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invariably stronger with response to fulvestrant. Anastrozole response was specifically correlated with three genes, of which KCNK15 was estrogen deprivation–related and SKP2 had predictive significance independent of pretreatment AURKA expression (Fig. 5B, iv). Further evaluation of the independence of the predictive performance of agent-specific genes was not conducted because of limited numbers.

Validation of differentially affected transcripts

qRT-PCR of clinical samples confirmed significant upregulation of CAV1 (1.87-fold increase, \( P = 0.0095 \)) and SNAI2 (1.88-fold increase, \( P = 0.0005 \)) by fulvestrant, whereas changes induced by anastrozole were not significant. The differential impact of the two agents on treatment-related transcripts (\( n = 46 \)) was consistent between the parent and bridging studies (Pearson \( r = 0.93, P < 0.0001, \) slope = 0.97). Fulvestrant-related transcripts (\( n = 41 \)) were concordantly affected, albeit to a lesser extent, following low-dose treatment (Pearson \( r = 0.77, P < 0.0001, \) slope = 0.23). Genes, which were found to respond differently to 4 weeks of fulvestrant and 2 weeks of anastrozole, were evaluated in a subgroup of patients receiving 16 weeks of anastrozole therapy. There was no significant change in any of the 11 transcripts differentially upregulated by fulvestrant, or 11 of the 17 transcripts differentially downregulated (Supplementary Table S13). This argues against the possibility that duration of treatment may have biased the identification of alterations in gene expression in favor of fulvestrant.

ESR1 knockdown in MCF7 cells

Knockdown of ESR1 invariably downregulated the expression of genes which were found to be differentially downregulated by fulvestrant (e.g., LRP8 and GTSE1) and upregulated the expression of some genes which were differentially upregulated by fulvestrant (e.g., SNAI2 and SEPP1), but not others (e.g. CAV1 and RBMS1). The majority of genes responded concordantly to fulvestrant and ESR1 knockdown (Supplementary Fig. S3).

Discussion

This study compared, for the first time, transcriptional profiles from breast cancer in situ following fulvestrant or anastrozole and corresponding in vitro models. The robust integrative strategy avoids the identification of spurious genes in clinical samples which may reflect increasing proportions of stroma with treatment response (33). The approach taken may discard alterations inadequately modeled in vitro, including those dependent upon three-dimensional structure, stromal interactions, and hypoxia, but focuses on those genes which may be subjected to functional interrogation in model systems. The hallmark molecular responses to interrupted estrogenic signaling, including

Figure 4. Network analysis of genes differentially affected by fulvestrant. Up- and downregulated transcripts are indicated in red and green, respectively. Direct and indirect relationships are indicated by solid and interrupted lines, respectively.
suppression of ERGs and proliferative markers, followed both fulvestrant and estrogen deprivation. However, distinguishing features were apparent. First, the overall transcriptional response to fulvestrant was of greater magnitude. Second, differences were not distributed uniformly across the transcriptome, but were most marked in a relatively limited cohort of genes.

A small number of differentially affected genes were specific to estrogen deprivation, potentially attributable to ERα-independent estrogen activity (34). Most were fulvestrant specific and remained unaffected by extended anastrozole treatment, raising the possibility of regulation by unliganded ERα. Both fulvestrant and estrogen deprivation abrogate estrogen-dependent ERα activity, but only fulvestrant, by virtue of ERα depletion, antagonizes estrogen-independent ERα activity, including cross-talk with growth factor pathways (26). The greater and differential transcriptional response to fulvestrant may be attributable to the arrest of estrogen-independent ERα activity that is unaffected by estrogen deprivation (27). This greater antiestrogenic effect is consistent with the greater efficacy of fulvestrant in studies comparing the agents as first-line therapy in advanced disease (16) and in its sequential utility after aromatase inhibitor relapse (9, 10). Incompletely overlapping transcriptional responses are also consistent with the reported efficacy of combination therapy with fulvestrant and anastrozole (35).

Genes differentially affected by fulvestrant were associated in networks with ERα, AR, and TP53. ERα activity can be influenced by cross-talk with AR signaling (36), which may exert antiestrogenic/antiproliferative effects in ERα-positive breast cancer, while having contrasting roles in ERα-negative tumors (37). The discovery of a subset of DNA-binding elements and pioneer factors common to both receptors (38) raises the possibility that activity of AR-dependent networks may be influenced by fulvestrant.

Figure 5. A, summary of treatment-related genes for which pretreatment expression correlates significantly with treatment-induced changes in expression of the proliferation-associated gene AURKA. Anastrozole (A, blue), fulvestrant (F, red), or both (green), with agent-specific and quantitative differences indicated by horizontal and vertical stripes, respectively. B, scattergrams illustrating the correlation between change in AURKA expression and three fulvestrant-specific genes (i)–(iii), and one estrogen deprivation-specific gene (iv), with predictive significance (P < 0.05) independent of pretreatment AURKA expression in NEWEST (red) and FAIMoS (blue).
induced loss of the ERα transcriptional program. Transcripts differentially upregulated by ERα depletion, including CAV1/2 and SNAI2, are associated with AR signaling and the biology of ERα-negative and basal-like breast cancer (39–42). CAV1 encodes caveolin-1, the principal constituent of specialized membrane invaginations called caveolae. Caveolin-1 and -2 are widely expressed and may colocalize. Caveolae have diverse functions, including: vesicular trafficking, lipid homeostasis, subcellular partitioning, and integrating the activity of signaling molecules. Caveolin-1 may facilitate nongenomic/extranuclear and ligand-independent ERα activity (42, 43). SNAI2 encodes SLUG, a transcription factor implicated in breast cancer progression, nodal involvement, and metastasis (44, 45). Expression is associated with epithelial-to-mesenchymal transition, E-cadherin downregulation, and stem cell-associated gene expression (46, 47). NRP1, encoding neuropilin-1, is a receptor for semaphorins and VEGF, also has links with stem cell phenotype (48) and poor prognosis (49).

Pretreatment expression of fulvestrant-related genes may be influenced by, and reflect, inherent tumor proliferation and/or estrogenicity. The possible agent-specific predictive utility of the differentially affected genes identified warrants further study. This may indicate whether such genes contribute mechanistically to the action of the two agents, are inconsequentially associated with treatment, and/or have external validity as predictive biomarkers. The impact of ESR1 knockdown on the expression of genes differentially affected by fulvestrant supports ERα destabilization as the mechanism underpinning particular fulvestrant-induced alterations. The identification of such genes may facilitate the comparative pharmacology of SERDs in development. This study also provides the first comparison of transcriptional responses with fulvestrant dosing regimens, and supports the efficacy of low-dose treatment (8–10). The greater transcriptional impact of high-dose therapy is consistent with pharmacokinetic models predicting 5-fold greater plasma concentrations on day 28 (50), and the increased efficacy observed in the NEWEST (17) and CONFIRM trials (13, 14).

Limitations of this study include in vitro modeling using a single cell line, which is also PI3KCA mutated, and expression profiling across different BeadChip versions which reduced the number of comparable probes. Potential confounding factors also include better patient compliance with treatment regimens in favor of fulvestrant given its mode of administration (although good estrogen-suppression was found in the aromatase inhibitor-treated patients—data not shown) and duration of treatment; extended anastrozole treatment may have induced further changes in gene expression. Nonrandomized comparisons may also be influenced by selection bias, with differences in baseline patient and tumor characteristics between presurgical studies with incompletely overlapping entry criteria, and differences such as details in sample taking, storage, and ethnicity of the populations. Sample sizes may have also restricted the statistical power to identify treatment-induced changes.

In conclusion, the molecular response to fulvestrant has much in common with estrogen deprivation, but is stronger with distinctions potentially attributable to arrest of estrogen-independent ERα activity and involvement of AR signaling. Genes responding differently to fulvestrant may have agent-specific predictive utility. These data are consistent with the efficacy of first-line fulvestrant versus anastrozole in advanced disease, combination therapy in the metastatic setting, sequential utility after aromatase inhibitor relapse, and higher dosing regimens.

Disclosure of Potential Conflicts of Interest

H. Anderson has ownership interests in AstraZeneca. M. Dowsett is a consultant/advisory board member for and reports receiving a commercial research grant and speakers’ bureau honoraria from AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


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References


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