Molecular Changes in Lobular Breast Cancers in Response to Endocrine Therapy


Abstract

Invasive lobular carcinoma (ILC) accounts for approximately 10% to 15% of breast carcinomas, and although it responds poorly to neoadjuvant chemotherapy, it appears to respond well to endocrine therapy. Pre- and on-treatment (after 2 weeks and 3 months) biopsies and surgical samples were obtained from 14 postmenopausal women with estrogen receptor–positive (ER+) histologically confirmed ILC who responded to 3 months of neoadjuvant letrozole and were compared with a cohort of 14 responding invasive ductal carcinomas (IDC) matched on clinicopathologic features. RNA was extracted and processed for whole human genome expression microarray. Dynamic clinical response was assessed using periodic three-dimensional ultrasound measurements performed during treatment and defined as a reduction of >70% in tumor volume by 3 months. Pretreatment profiles of ILC and IDC tumors showed distinctive expression of genes associated with E-cadherin signaling, epithelial adhesion, and stromal rearrangement. The changes in gene expression in response to letrozole were highly similar between responding ILC and IDC tumors; genes involved in proliferation were downregulated and those involved with immune function and extracellular matrix remodeling were upregulated. However, molecular differences between the histologic subtypes were maintained upon treatment. This is the first study of molecular changes in ILC in response to endocrine therapy to date. The genes that change on letrozole are highly consistent between ILC and IDC. Differences in gene expression between ILC and IDC at diagnosis are maintained at each time point on treatment. Cancer Res; 74(19); 5371-6. ©2014 AACR.

Introduction

Invasive lobular carcinoma (ILC) accounts for approximately 10% to 15% of newly diagnosed breast cancers (1, 2), affecting roughly 30,000 women annually in the United States (2). ILC is classically characterized by small, regular uniform neoplastic cells that invade the stroma in a single-file pattern with cells encircling normal breast tissue (1). Inactivation of E-cadherin (CDH1) by a variety of molecular mechanisms is considered a defining characteristic of ILC. When compared with the more common invasive breast cancers of no special type, also known as invasive ductal carcinomas (IDC), ILC is more likely to be estrogen receptor–positive (ER+) and of lower nuclear grade (3). ILC is often large at diagnosis and there have been numerous reports on the response to neoadjuvant chemotherapy. It was thought that patients with ILC are significantly less likely to have a pathologic complete response than patients with IDC; however, a recent study concluded that ILC represents a heterogeneous group of tumors and the difference in response to neoadjuvant chemotherapy is largely explained by differences in molecular characteristics, particularly hormone receptor and HER2, and is independent of lobular histology (4). ILC have been shown to respond well to endocrine therapy (2) and we recently described the clinical response to neoadjuvant letrozole in a series of 61 patients (3). The lack of understanding of how lobular breast cancer responds to treatment is compounded by the paucity of research models (reviewed in ref. 2); however, a very recent study suggested that E2 and anti-estrogens differentially regulate ERα-mediated gene expression in ILC versus IDC cell lines and xenografts (5).

Previous microarray studies of ILC tumors have focused on transcriptional differences between lobular and ductal histology before treatment (6–10). To our knowledge, there have been no previous studies on the molecular response to endocrine therapy in ILC patient samples. We and others have characterized the molecular response to endocrine therapy in breast tumors in previous studies (11–13), but have not previously considered the effects of histologic subtype. Comparing pre- and posttreatment biopsies from the same patients, using the "window of opportunity" afforded with neoadjuvant therapy is a powerful approach that can improve statistical power due to reducing patient–patient variation (14). However, these studies are challenging to perform and are dependent on analyzing sufficient numbers of suitably appropriate samples.

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In this study, we have performed the first gene expression profiling study of ILC treated with neoadjuvant letrozole and compared the molecular response with that of IDC.

Patients and Methods

Patients

Samples were selected from a consecutive series of 89 postmenopausal women presenting to the Edinburgh Breast Unit (Western General Hospital, Edinburgh, United Kingdom) between 2003 and 2011. Each had a large primary histologically confirmed invasive breast cancer, immunohistochemically determined to be ER+*. All patients gave informed consent to be included in the study that was approved by the local regional ethics committee (2001/8/80 and 2001/8/81). Patients were treated within a neoadjuvant protocol, in which letrozole (Femara, 2.5 mg; Novartis Pharma AG) was given daily.

Tumor samples

Tumor biopsies were taken with a 14-guage needle before and approximately 2 weeks (range, 10–19 days) and 3 months (range, 86–142 days) following commencement of continuous letrozole treatment as described previously (Fig. 1A). Samples were snap-frozen in liquid nitrogen and frozen sections were taken, stained with hematoxylin and eosin (H&E), and the cellularity and percentage presence of cancerous tissue within each specimen were assessed by a pathologist. Two-week samples were available only for 10 of the 14 patients with ILC.

Response assessment

Clinical response was determined using dynamic changes in tumor volumes assessed by repeated measurements taken over the 3-month treatment period. Primary assessment was based on ultrasound measurements performed by a single
clinician (J.M. Dixon) and these were verified by mammographic measurements (Fig. 1A). Clinical response was defined as a reduction of greater than 70% in tumor volume by 3 months.

**RNA processing and microarray hybridization**

Biopsies were homogenized and RNA was extracted using the RNeasy Mini Kit with RNase-free DNase treatment (Qiagen). RNA quantity and quality were verified on a Bioanalyzer 2100 with the RNA 6000 Nano Kit (Agilent) and Nanodrop 2000c (Thermo Scientific). RNA was reverse transcribed and amplified using the WT-Ovation FFPE System Version 2 (NuGEN), purified using the Qiaquick PCR Purification Kit (Qiagen), biotinylated using the IL EnCore Biotin Module (NuGEN), purified using the minElute Reaction Cleanup Kit (Qiagen) and quantified once again using the Nanodrop 2000c (Thermo Scientific). Labeled cDNA was hybridized to Human HT-12v4 whole-genome expression bead arrays (Illumina) according to the standard protocol for NuGEN amplified samples. The Illumina data have been submitted to NCBI Gene Expression Omnibus (GEO) and are available under GSE55374. Approximately half of the ILC and IDC samples were processed on Affymetrix GeneChips within a previous study (12), these are publicly available from NCBI GEO under GSE20181 and identified in the Supplementary Data.

**Data analysis**

The Illumina and Affymetrix data were independently pre-processed and re-annotated to Ensembl gene identifiers, then combined and batch corrected as described previously (15). Briefly, Illumina probe profiles were quantile normalized using the *lumi* package and mapped to Ensembl gene sequences using reMOAT (16), BioMart, and a custom BLAST sequence search. A custom Chip Definition File (CDF; ref. 17) was used to map the Affymetrix data to Ensembl. The Affymetrix portion of data was normalized by robust multi-array average method implemented by the *affy* package. The datasets were filtered using detection *P* values, removing probes that were undetected (*P* > 0.05 in the total minus three samples). Both datasets were then combined and batch corrected with cross-platform normalization (XPN; ref. 18). A subset of samples was profiled on both platforms and demonstrated successful minimization of batch effects (15). Pretreatment tumors were assigned to molecular subtypes using the Sorlie and colleagues centroids (19) as described previously (15). Paired and unpaired Rank Products analysis (20) was used to identify differentially expressed genes and gene set enrichment analysis (GSEA) was performed with the *phenoTest* package. Functional gene ontology analysis was performed using DAVID Bioinformatics Resources 6.7 and the PANTHER Classification System. Differences in clinicopathologic features and platforms between the ILC and IDC samples were assessed with the *χ²* test.

**Results**

From a cohort of 61 patients with ILC treated with neoadjuvant letrozole (3), surgery was possible for 24 cancers after 3 months. Sufficient quality and quantity of RNA for gene expression profiling were available for matched pretreatment and 3-month samples for 14 of these patients with ILC who had a clinical response to letrozole. Pre- and 3-month letrozole-treated transcriptome data were also selected for a further 14 patients with IDC that responded to letrozole. Patients were matched for clinicopathologic features (Table 1) and response (Fig. 1B) and the histopathologic status were confirmed by a pathologist (representative images Fig. IC). Consistent with previous studies (6–10), unsupervised hierarchical clustering of the pretreatment samples using the 500 most variable genes across samples at pretreatment was able to distinguish between IDC and ILC with 86% accuracy (Fig. 1D).

**Molecular differences between ILC and IDC are maintained on treatment**

Supervised analysis [Rank Products, percent false present (PFP) = 0.05] identified 206 genes differentially expressed between the histologic subtypes before treatment. The 70 genes that had lower levels of expression in ILC than IDC tumors were functionally enriched for immune and extracellular matrix (ECM) remodeling genes, including several genes that have been highlighted in previous studies, including E-cadherin (CDH1), osteopontin (SPP1), and epithelial cell adhesion molecule (EPCAM). Similarly, many of the 136 genes

<table>
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<th>Lobular breast cancers (14)</th>
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**Table 1. No significant difference in clinicopathologic features or platform of ILC and IDC tumors**
with significantly higher expression in ILC than IDC have previously been shown to distinguish between these histologic subtypes (7–10), and include CAV1, AOC3, FAB4, VWF, TF, CD36, EGR1, IER2, and PLIN1. Over half of the genes found to be significantly differentially expressed between the histologic subtypes before treatment (including all of those highlighted) were still significantly differentially expressed after 3 months of treatment (Fig. 2A and B). GSEA confirmed consistency of the gene lists before and after treatment (P < 0.0001). Therefore, differences in gene expression between ductal and lobular carcinomas are maintained during treatment, as illustrated using multidimensional scaling of the 206 pretreatment genes with time on the third (horizontal) axis showing that differences in gene expression between ILC and IDC are maintained. Red spots represent ILC and blue represent IDC biopsies and surgical samples.

**Highly similar molecular response to letrozole in ILC and IDC**

Gene expression profiles of surgical samples after 3 months of letrozole treatment were compared with their representative patient-matched pretreatment biopsy samples using a pairwise Rank Products analysis (PFP, 0.05) for patients with ILC and IDC. Over half of the changed genes were significantly up- or downregulated in both histologic subtypes. Figures 3A and B clearly demonstrates that the molecular effects of treatment are virtually uniform (even after just 2 weeks), in the two subtypes, with the same genes up- and downregulated. This result is somewhat at odds with a very recent study that found that E2 and anti-estrogens differentially regulate ERα-mediated gene expression in ILC (MDA-MB-134VI and SUM44PE) and IDC (MCF7, T47D, and BT474) cell lines (5). However, we found that the “ILC-specific” and “IDC-specific” genes identified in this study were not significantly changed in the clinical samples after neoadjuvant letrozole using GSEA to compare the response between the histologic subtypes (Supplemental Data). Furthermore, none of the differentially regulated genes highlighted in ILC cell lines and represented in our data (CA12, NEDD9, CXCL12, PDE4B, and NR3C2) were significantly differently regulated between ILC and IDC tumors treated with letrozole (Supplemental Data). Perhaps not
surprisingly and consistent with previous studies (12, 13), the
genes that were most signifi-
cantly changed in response to
letrozole in both ILC and IDC tumors were characterized by
downregulation of proliferation and upregulation of ECM
remodeling and immune pathways (Fig. 3C).

Discussion
Our study shows for the first time that the molecular
response to endocrine therapy in ILCs is highly similar to the
response in IDCs. This is somewhat surprising given that we
and others (7–10) have demonstrated clear molecular dif-
ferences between tumors before treatment. The present
study demonstrates that these differences are maintained
during treatment. The present study demonstrates that these differences are maintained
during treatment. The consistent molecular changes in expression observed in
response to letrozole in both ILC and IDC contrast with a
recent study that found that E2 and anti-estrogens differ-
tentially regulate ERα-mediated gene expression in ILC and
IDC cell lines (5). Although there are obvious possible
explanations for this apparent discrepancy, including differ-
tent responses to alternative endocrine agents and the degree
to which a small number cell lines represent the molecular
heterogeneity of primary breast tumors, we believe that our
study demonstrates the value and need for performing
molecular studies in patient samples undergoing treatment,
rather than in cell lines. One of the possible reasons behind
the similar molecular response to treatment observed in
ILC and IDC in this study is that we selected only clearly
responsive tumors from both histologic subtypes.

Previous work from our group has suggested that there is
greater molecular diversity in the gene changes seen
between individual nonresponding tumors when treated
with aromatase inhibitors, whereas in responders the
changes are relatively homogeneous (12). Thus, the resis-
tance mechanisms still may be different between the ILC and
IDC despite the similarities found in responding tumors in
the present study. We were unable to address this issue at
this time, as there were only two nonresponding lobular
tumors with microarray data and therefore they were not
included in this study. An extension of this study focused
on the different resistance pathways in nonresponding

Figure 3. Comparison of changes in ILC and IDC treated with letrozole. A, Venn diagrams showing the overlapping genes that are consistently up- and down-
regulated in response to letrozole in ILC and IDC, comparing 3-month and pretreatment samples by pairwise Rank Products analysis (PFP, 0.05). B, heatmap
showing consistent, ILC- and IDC-specific changes. The colors represent changes in gene expression, up- (red) and downregulated genes, relative to
pretreatment patient-matched samples, respectively. C, pie charts showing genes downregulated (top) and upregulated (bottom) at 3 months, grouped by
functional association. Each gene was assigned to only one group that was the most significantly enriched functional process or pathway as determined by
DAVID Bioinformatics Resources 6.7 and the PANTHER Classification System.
tumors of different pathologic types may be possible in the future if additional samples can be collected. However, we have already recorded that some nonresponding tumors have molecular changes similar to responders; notably, reductions in estrogen-regulated and proliferation genes, without a clear clinical response (21).

Although the number of patients in this study is relatively modest, the ILC and IDC patient groups consist of cancers with a consistently high Allred ER score of 7 or 8, all were luminal A subtype, grade 2 or 3, and had similar responses to letrozole. Relatively high numbers of genes were significantly differentially expressed between the two subtypes, demonstrating clear distinctions between these cancers that were maintained at all time points. The study also supports the potential value of cross-platform integration to generate larger datasets with increased statistical power given that clinical samples are relatively scarce.

In conclusion, we have performed the first study of molecular changes in ILC in response to endocrine therapy. The genes that change on letrozole treatment are highly similar in ILC and IDC although clear molecular differences between the histologic subtypes are evident between these two cancer types, and these differences are maintained on treatment.

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

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.M. Arthur, A.K. Turnbull, V.L. Webber, L. Renshaw, C. Kay, J.S. Thomas
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.M. Arthur, A.K. Turnbull, J.M. Dixon, A.H. Sims
Writing, review, and/or revision of the manuscript: L.M. Arthur, A.K. Turnbull, A.A. Larionov, J.S. Thomas, J.M. Dixon, A.H. Sims
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Renshaw, J.M. Dixon, A.H. Sims
Study supervision: A.K. Turnbull, J.M. Dixon, A.H. Sims

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References
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