

Molecular Changes in Lobular Breast Cancers in Response to Endocrine Therapy

Laura M. Arthur, Arran K. Turnbull, Victoria L. Webber, Alexey A. Larionov, Lorna Renshaw, Charlene Kay, Jeremy S. Thomas, J. Michael Dixon, and Andrew H. Sims

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); 1–6. ©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 ER α -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.

Edinburgh Breast Unit, University of Edinburgh, Western General Hospital, Edinburgh, United Kingdom.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Corresponding Authors: Andrew H. Sims, Edinburgh Breast Unit, University of Edinburgh, Western General Hospital, Crewe Road South, Edinburgh, EH4 2XR, United Kingdom. Phone: 44-131-777-3516; Fax: 44-131-777-3520; E-mail: andrew.sims@ed.ac.uk; and J. Michael Dixon, mike.dixon@ed.ac.uk

doi: 10.1158/0008-5472.CAN-14-0620

©2014 American Association for Cancer Research.

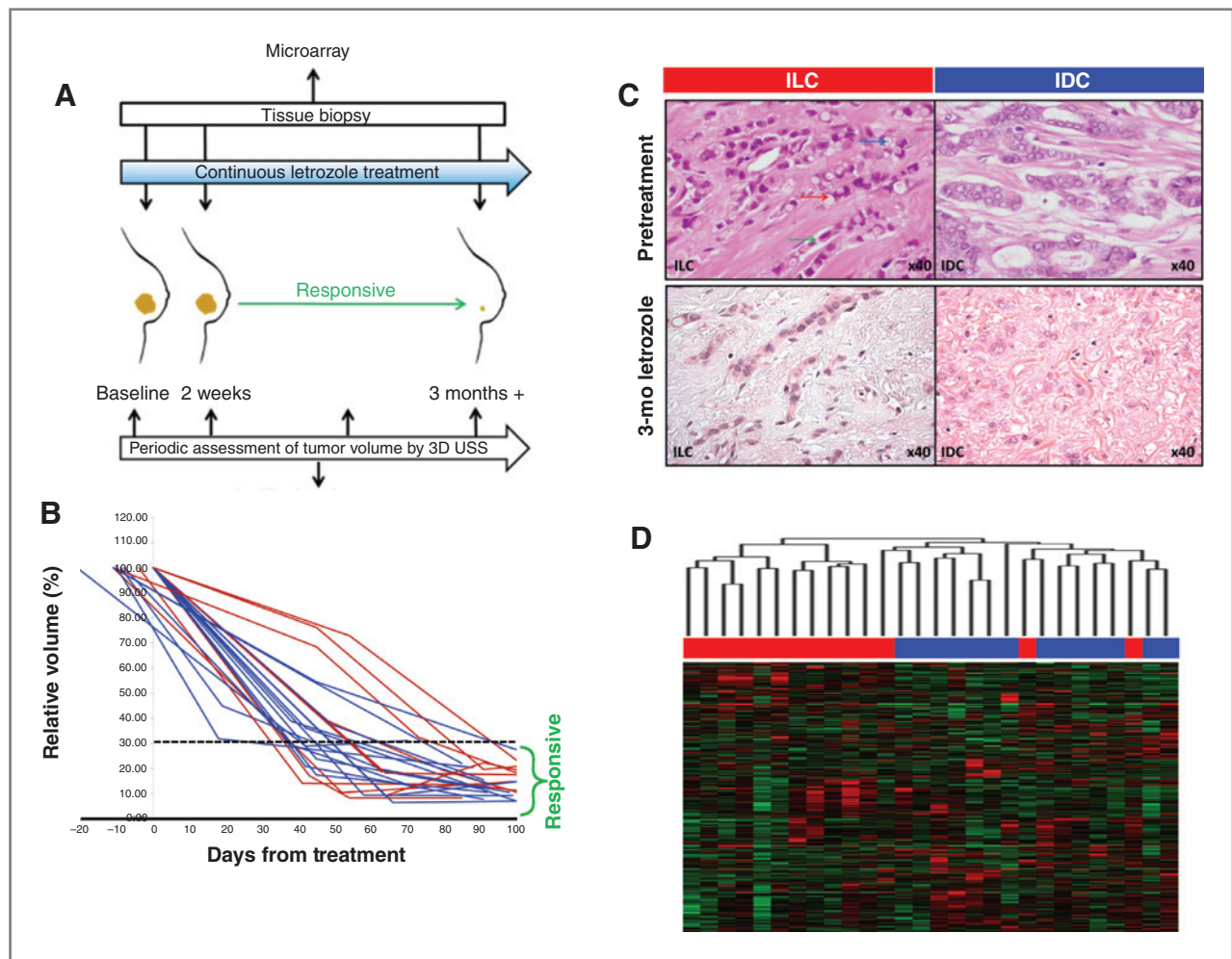


Figure 1. Lobular and ductal breast carcinomas have similar response patterns to letrozole, but have distinct molecular profiles, reflecting differences in histology. **A**, schematic showing biopsies taken before and after 2 weeks and 3 months on letrozole treatment. **B**, tumor volume ultrasound changes in the 14 ILC (red) and 14 IDC (blue). **C**, representative images of ILC (left) and IDC (right). ILC is characterized by cancer cells invading the stroma in single file patterns. Blue arrow, cytoplasmic eosinophilic inclusions; red arrow, vacuolated cytoplasm; and green arrow, the clear spaces between adjacent cells. **D**, unsupervised hierarchical clustering heatmap of the 500 most variable genes across the 14 ILC and 14 IDC pretreatment. Colors represent relative differences in \log_2 mean-centered gene expression. Red denotes higher expression and green lower expression. The color bar between the clustering tree and the heatmap indicates whether the tumors are ILC (red) or IDC (blue).

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-gauge 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 χ^2 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

Table 1. No significant difference in clinicopathologic features or platform of ILC and IDC tumors

	Lobular breast cancers (14)	Ductal breast cancers (14)	<i>P</i>
Grade			0.21
1	0	0	
2	9	13	
3	3	1	
ER (Allred) score			0.66
7	3	4	
8	11	10	
HER2			1
Negative	13	13	
Positive	1	1	
3-month sample			0.16
Surgical resection	13	11	
Core biopsy	1	3	
Intrinsic subtype			1
Luminal A	14	14	
Tumor size			0.48
T1	1	0	
T2	6	10	
T3	1	1	
T4	5	3	
Nodes			0.52
Negative	9	8	
Positive	4	6	
Microarray platform			0.70
Affymetrix	7	8	
Illumina	7	6	

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. 1C). 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

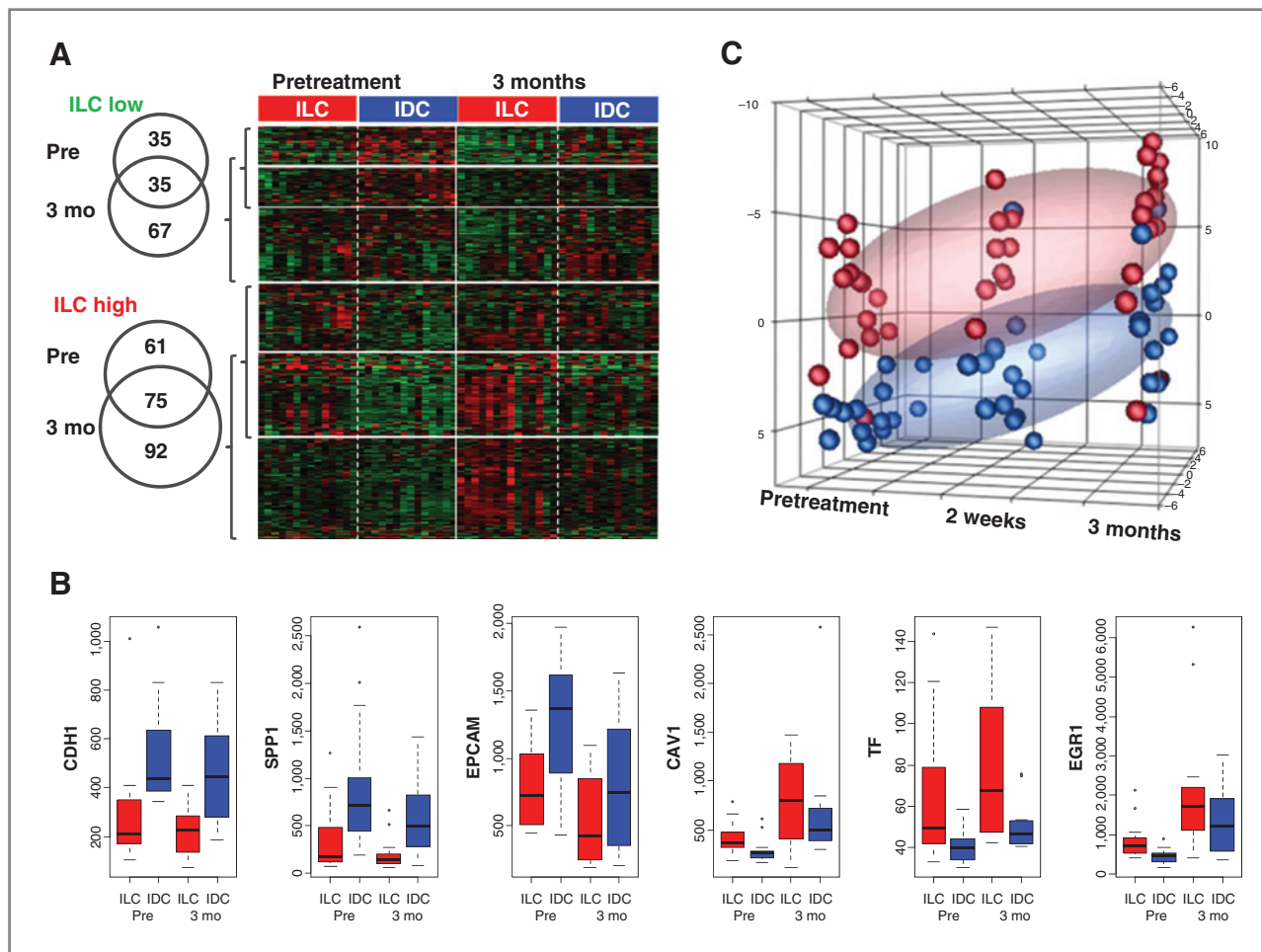


Figure 2. Differences between ILC and IDC are maintained on treatment. **A**, Venn diagrams and heatmap showing overlap using the same criteria (Rank Products, PFP = 0.05) at baseline and 3 months. Heatmap colors show log₂ mean-centered values; red, high expression; green, low expression. **B**, boxplots for genes previously identified to be differentially expressed between ILC and IDC; red, ILC; blue, IDC. **C**, multidimensional scaling plot 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.

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 plotted on the third dimension (Fig. 2C).

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 ER α -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

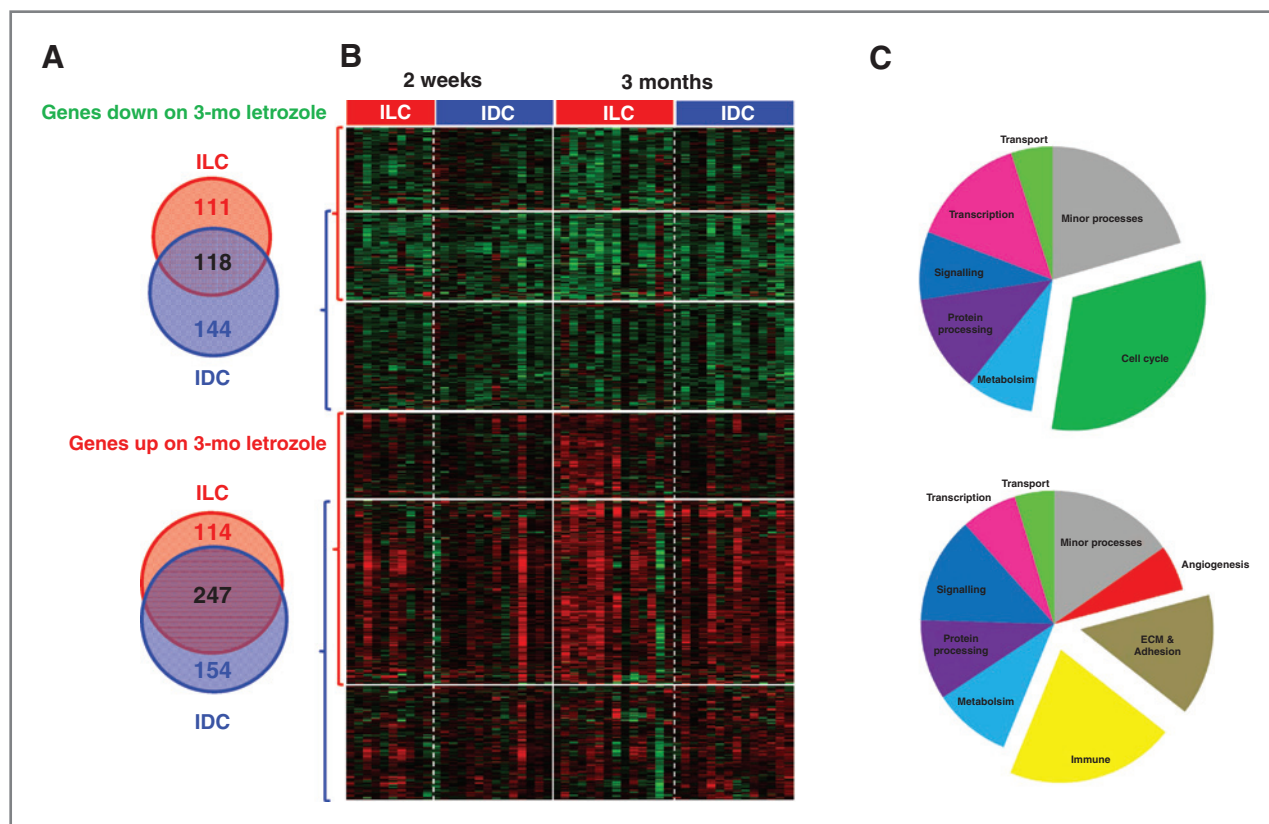


Figure 3. Comparison of changes in ILC and IDC treated with letrozole. A, Venn diagrams showing the overlapping genes that are consistently up- and down-regulated in response to letrozole in ILC and IDC, comparing 3-month and pretreatment samples by pairwise Rank Products analysis (PFP, 0.05). B, heatmap 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.

surprisingly and consistent with previous studies (12, 13), the genes that were most significantly 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 differences between tumors before treatment. The present study demonstrates that these differences are maintained during treatment. Stratified medicine seeks to identify molecular differences between cancers that will allow targeted treatment with specific agents, yet although these histologic subtypes of breast cancer are molecularly distinct, both respond in a highly uniform way to endocrine therapy. 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 differentially regulate ER α -mediated gene expression in ILC and

IDC cell lines (5). Although there are obvious possible explanations for this apparent discrepancy, including different 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 resistance 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

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.

References

- Dixon JM, Anderson TJ, Page DL, Lee D, Duffy SW. Infiltrating lobular carcinoma of the breast. *Histopathology* 1982;6:149-61.
- Sikora MJ, Jankowitz RC, Dabbs DJ, Oesterreich S. Invasive lobular carcinoma of the breast: patient response to systemic endocrine therapy and hormone response in model systems. *Steroids* 2013; 78:568-75.
- Dixon JM, Renshaw L, Dixon J, Thomas J. Invasive lobular carcinoma: response to neoadjuvant letrozole therapy. *Breast Cancer Res Treat* 2011;130:871-7.
- Lips EH, Mukhtar RA, Yau C, de Ronde JJ, Livasy C, Carey LA, et al. Lobular histology and response to neoadjuvant chemotherapy in invasive breast cancer. *Breast Cancer Res Treat* 2012;136:35-43.
- Sikora MJ, Cooper KL, Bahreini A, Luthra S, Wang G, Chandran UR, et al. Invasive lobular carcinoma cell lines are characterized by unique estrogen-mediated gene expression patterns and altered tamoxifen response. *Cancer Res* 2014;74:1463-74.
- Turashvili G, Bouchal J, Baumforth K, Wei W, Dziechciarkova M, Ehrmann J, et al. Novel markers for differentiation of lobular and ductal invasive breast carcinomas by laser microdissection and microarray analysis. *BMC Cancer* 2007;7:55.
- Korkola JE, DeVries S, Fridlyand J, Hwang ES, Estep AL, Chen YY, et al. Differentiation of lobular versus ductal breast carcinomas by expression microarray analysis. *Cancer Res* 2003;63:7167-75.
- Bertucci F, Orsetti B, Negre V, Finetti P, Rouge C, Ahomadegbe JC, et al. Lobular and ductal carcinomas of the breast have distinct genomic and expression profiles. *Oncogene* 2008;27:5359-72.
- Weigelt B, Geyer FC, Natrajan R, Lopez-Garcia MA, Ahmad AS, Savage K, et al. The molecular underpinning of lobular histological growth pattern: a genome-wide transcriptomic analysis of invasive lobular carcinomas and grade- and molecular subtype-matched invasive ductal carcinomas of no special type. *J Pathol* 2010;220:45-57.
- Zhao H, Langerod A, Ji Y, Nowels KW, Nesland JM, Tibshirani R, et al. Different gene expression patterns in invasive lobular and ductal carcinomas of the breast. *Mol Biol Cell* 2004;15:2523-36.
- Mackay A, Urruticoechea A, Dixon JM, Dexter T, Fenwick K, Ashworth A, et al. Molecular response to aromatase inhibitor treatment in primary breast cancer. *Breast Cancer Res* 2007; 9:R37.
- Miller WR, Larionov A, Renshaw L, Anderson TJ, Walker JR, Krause A, et al. Gene expression profiles differentiating between breast cancers clinically responsive or resistant to letrozole. *J Clin Oncol* 2009; 27:1382-7.
- Dunbier AK, Ghazoui Z, Anderson H, Salter J, Nerurkar A, Osin P, et al. Molecular profiling of aromatase inhibitor-treated post-menopausal breast tumors identifies immune-related correlates of resistance. *Clin Cancer Res* 2013;19:2775-86.
- Sims AH, Bartlett JM. Approaches towards expression profiling the response to treatment. *Breast Cancer Res* 2008;10:115.
- Turnbull AK, Kitchen RR, Larionov A, Renshaw L, Dixon JM, Sims AH. Direct integration of intensity-level data from Affymetrix and Illumina microarrays improves statistical power for robust reanalysis. *BMC Med Genomics* 2012;5:35.
- Barbosa-Morais NL, Dunning MJ, Samarajiva SA, Darot JF, Ritchie ME, Lynch AG, et al. A re-annotation pipeline for Illumina BeadArrays: improving the interpretation of gene expression data. *Nucleic Acids Res* 2009;38:e17.
- Dai M, Wang P, Boyd AD, Kostov G, Athey B, Jones EG, et al. Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. *Nucleic Acids Res* 2005;33:e175.
- Shabalina AA, Tjelmeland H, Fan C, Perou CM, Nobel AB. Merging two gene-expression studies via cross-platform normalization. *Bioinformatics* 2008;24:1154-60.
- Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A* 2003;100:8418-23.
- Breitling R, Armengaud P, Amtmann A, Herzyk P. Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. *FEBS Lett* 2004;573:83-92.
- Miller WR, Larionov A. Changes in expression of oestrogen regulated and proliferation genes with neoadjuvant treatment highlight heterogeneity of clinical resistance to the aromatase inhibitor, letrozole. *Breast Cancer Res* 2010;12:R52.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: L.M. Arthur, A.K. Turnbull, J.M. Dixon, A.H. Sims
Development of methodology: L.M. Arthur, A.K. Turnbull, A.A. Larionov, J.M. Dixon, A.H. Sims
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

Acknowledgments

The authors are also grateful to the Experimental Cancer Medicine Centre (ECMC; Western General Hospital) for tissue storage and retrieval and to Robert Morris, Susan Harvey, and Helen Caldwell for sectioning of frozen and formalin-fixed paraffin-embedded tissues and H&E staining.

Grant Support

This study was financially supported by Breakthrough Breast Cancer, Breast Cancer Research Trust, and Melville Trust.

Received February 28, 2014; revised June 10, 2014; accepted July 2, 2014; published OnlineFirst August 6, 2014.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Molecular Changes in Lobular Breast Cancers in Response to Endocrine Therapy

Laura M. Arthur, Arran K. Turnbull, Victoria L. Webber, et al.

Cancer Res Published OnlineFirst August 6, 2014.

Updated version	Access the most recent version of this article at: doi: 10.1158/0008-5472.CAN-14-0620
Supplementary Material	Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2014/08/06/0008-5472.CAN-14-0620.DC1

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org .
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org .