Molecular changes in lobular breast cancers in response to endocrine therapy

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ABSTRACT (229)

Invasive lobular cancer (ILC) accounts for approximately 10-15% 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 post-menopausal women with ER+ histologically confirmed ILC who responded to 3 months of neoadjuvant letrozole and were compared with a cohort of 14 responding infiltrating ductal carcinomas (IDCs) matched on clinicopathological features. RNA was extracted and processed for whole human genome expression microarray. Dynamic clinical response was assessed using periodic 3D ultrasound measurements performed during treatment and defined as a reduction of >70% in tumour volume by 3 months.

Pre-treatment profiles of ILC and IDC tumours showed distinctive expression of genes associated with e-cadherin signalling, epithelial adhesion and stromal rearrangement. The changes in gene expression in response to letrozole were highly similar between responding ILC and IDC tumours, genes involved in proliferation were down-regulated and those involved with immune function and ECM remodelling were up-regulated. However, molecular differences between the histological subtypes were maintained upon treatment. This is the first study of molecular changes in ILC in response to endocrine therapy to date. The genes which 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.
INTRODUCTION

Invasive lobular carcinoma (ILC) accounts for approximately 10-15% 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 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 pathological complete response than IDC patients, however a recent study concluded that ILC represents a heterogeneous group of tumours and the difference in response to neoadjuvant chemotherapy is largely explained by differences in molecular characteristics, particularly HR 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 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 tumours 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 characterised the molecular response to endocrine therapy in breast tumours in previous studies (11-13), but have not previously considered the effects of histological subtype. Comparing pre- and post-treatment biopsies from the same patients, utilising the ‘window of opportunity’ afforded with neoadjuvant therapy is a powerful approach which can improve statistical power due to reducing patient-patient variation (14). However these studies are challenging to perform and are dependent on analysing sufficient numbers of suitably appropriate samples.

In this study we have performed the first gene expression profiling study of ILC treated with neoadjuvant letrozole and compare the molecular response to that of IDC.
Materials and Methods

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

Tumour samples
Tumour 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 (Figure 1A). Samples were snap-frozen in liquid nitrogen and frozen sections taken, stained with haematoxylin and eosin (H&E) and the cellularity and percentage presence of cancerous tissue within each specimen was assessed by a pathologist. Two week samples were available only for 10 of the 14 ILC patients.

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

RNA Processing and microarray hybridisation
Biopsies were homogenised and RNA was extracted using the RNaseasy Mini Kit with RNase-free DNase treatment (Qiagen). RNA quantity and quality was verified on a Bioanalyser 2100 with 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 minElute Reaction Cleanup Kit (Qiagen) and...
quantified once again using the Nanodrop 2000c (Thermo Scientific). Labelled cDNA was hybridised to Human HT-12v4 whole-genome expression beadarrays (Illumina) according to the standard protocol for NuGEN amplified samples. The Illumina data has been submitted to NCBI GEO and is 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 file.

**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 normalised 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) [17] was used to map the Affymetrix data to Ensembl. The Affymetrix portion of data was normalised by RMA 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 3 samples). Both datasets were then combined and batch corrected with cross-platform normalisation (XPN) [18]. A subset of samples was profiled on both platforms and demonstrated successful minimisation of batch effects [15]. Pre-treatment tumours were assigned to molecular subtypes using the Sorlie *et al.* 2003 centroids as described previously [15]. Paired and unpaired Rank Products analysis [19] 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 Resource 6.7 and the PANTHER classification system. Differences in clinicopathological features and platforms between the ILC and IDC samples were assessed with the Chi-Square test.

**Results**

From a cohort of sixty-one 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 was available for matched pre-treatment and 3 month samples for 14 of these ILC patients who had a clinical response to letrozole. Pre and 3 month letrozole-treated transcriptome data was also selected for a further 14 patients with IDC that
responded to letrozole. Patients were matched for clinicopathological features (Table 1) and response (Figure 1B) and the histopathological status was confirmed by a pathologist (representative images Figure 1C). Consistent with previous studies (6-10), unsupervised hierarchical clustering of the pre-treatment samples using the 500 most variable genes across samples at pre-treatment was able to distinguish between IDC and ILC with 86% accuracy (Figure 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 histological subtypes prior to treatment. The 70 genes that had lower levels of expression in ILC than IDC tumours were functionally enriched for immune and extra-cellular matrix (ECM) remodelling 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 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 the genes found to be significantly differentially expressed between the histological subtypes before treatment (including all of those highlighted) were still significantly differentially expressed after 3 months of treatment (Figure 2A and 2B). Gene set enrichment analysis 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 pre-treatment genes with time plotted on the third dimension (Figure 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 pre-treatment biopsy samples using a pair-wise Rank Products analysis (PFP=0.05) for ILC and IDC patients. Over half of the changed genes were significantly up or down regulated in both histological subtypes. Figure 3A 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 down-regulated. 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 histological subtypes (Supplemental Figure). 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 tumours treated with letrozole (Supplemental Figure). Perhaps not surprisingly and consistent with previous studies (12, 13), the genes that were most significantly changed in response to letrozole in both ILC and IDC tumours were characterised by down-regulation of proliferation and up-regulation of extracellular matrix remodelling and immune pathways (Figure 3B).

**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 tumours prior to 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 histological 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 ERα-mediated gene expression in ILC and IDC cell lines (5). Whilst 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 tumours, 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 tumours from both histological subtypes.

Previous work from our group has suggested that there is greater molecular diversity in the gene changes seen between individual non-responding tumours 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 tumours in the present study. We were unable to address this issue at this time, as there was only two non-responding lobular tumour with microarray data and therefore they were not included in this study. An extension of this study focused on the different resistance pathways in non-responding tumours of different pathological types may be possible in the future if additional samples can be collected. However, we have already recorded that some non-responding tumours have molecular changes similar to responders; notably reductions in oestrogen-regulated and proliferation genes, without a clear clinical response (20).

Whilst 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 which was 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 which change on letrozole treatment are highly similar in ILC and IDC although clear molecular differences between the histological subtypes are evident between these two cancer types, and these differences are maintained on treatment.

Acknowledgements

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References


Table 1. No significant difference in clinicopathological features or platform of ILC and IDC tumours

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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, Tumour volume ultrasound changes in the 14 ILC (red) and 14 IDC (blue). C, Representative images of ILC (left) and IDC (right), ILC is characterised by cancer cells invading the stroma in single file patterns Blue arrow indicates cytoplasmic eosinophilic inclusions, red arrow shows vacuolated cytoplasm and green arrow indicates the clear spaces between adjacent cells. D, Unsupervised hierarchical clustering heatmap of the 500 most variable genes across the 14 ILC and 14 IDC pre-treatment, colours represent relative differences in log2 mean centred gene expression. Red denotes higher expression and green lower expression. The colour bar between the clustering tree and the heatmap indicates whether the tumours are ILC (red) or IDC (blue).

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 colours show log2 mean-centred values, Red=high and 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 pre-treatment genes with time on the 3rd (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.

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 pre-treatment samples by pairwise Rank Products analysis (PFP=0.05). B, Heatmap showing consistent, ILC-specific and IDC-specific changes, the colours represent changes in gene expression, up (red) and down-regulated genes, relative to pre-treatment patient-matched samples respectively. C, Pie charts showing genes down-regulated (top) and up-regulated (bottom) at 3 months grouped by functional association. Each gene was assigned to only one group which was the most significantly enriched functional process or pathway as determined by DAVID Bioinformatics resource 6.7 and the PANTHER classification system.
Figure 1

Panel A: A flowchart showing the treatment process from Microarray to Tissue Biopsy, followed by Continuous Letrozole Treatment, and then Periodic Assessment of Tumor Volume by 3D USS. The treatment progresses from Baseline to 14-days and then 3-months +, with the green arrow indicating responsive outcomes.

Panel B: A graph showing the relative volume (%) over days from treatment, illustrating the decline in tumor size after treatment.

Panel C: Images comparing ILC (Invasive Lobular Carcinoma) and IDC (Invasive Ductal Carcinoma) pre-treatment and after 3 months of treatment. The images are labeled as x40.

Panel D: A heat map showing gene expression data, with red and green colors indicating upregulation and downregulation, respectively.
Figure 3

A

Genes down on 3m letrozole

ILC

111

118

144

IDC

Genes up on 3m letrozole

ILC

114

247

154

IDC

B

2 weeks

3 months

C

Transport

Transcription

Minor Processes

Cell cycle

Signalling

Protein Processing

Metabolism

Angiogenesis

Immune

HiM & Adhesion

Metabolism

Protein Processing

Signalling

Transcription
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