Changes in breast cancer biomarkers in the IGF1R/PI3K pathway in recurrent breast cancer after tamoxifen treatment

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Abstract

Development of resistance to the anti-oestrogen tamoxifen occurs in a large proportion of patients with oestrogen receptor-positive (ER+) breast cancer and is an important clinical challenge. While loss of ER occurs in c.20% of tamoxifen-resistant tumours, this cannot be the sole explanation for tamoxifen treatment failure. PI3K pathway activation, including by insulin-like growth factor receptor 1 (IGF1R), has been implicated in some resistance models. The primary aim was to determine whether evidence exists in clinical breast cancer for a role of IGF1R and/or the PI3K pathway, in acquisition of resistance to tamoxifen. Invasive primary and recurrent tamoxifen-resistant tumours from the same patient (n=77) were assessed for changes in ER, progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2), IGF1R, stathmin, PTEN expression and PIK3CA mutations where possible. ER and PgR levels were significantly reduced at recurrence with 22 and 45%, respectively, showing negative status at this time. Acquisition of HER2 overexpression occurred in 6% of cases. IGF1R expression was significantly reduced in both ER+ and ER− recurrences and stathmin levels increased. A positive association between stathmin and IGF1R emerged in recurrent samples, despite their opposing relationships with ER, suggesting some coalescence of their activities may be acquired. The data confirm loss of ER and PgR and gain of HER2 in some tamoxifen-resistant tumours. There is no evidence for IGF1R gain in tamoxifen resistance, but increases in stathmin levels suggest that activation of the PI3K pathway may have contributed, but PTEN loss and PIK3CA hotspot mutations were relatively rare.

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Introduction

Until recently, tamoxifen has been the gold standard endocrine treatment for oestrogen receptor-positive (ER+) breast cancer and despite the advent of aromatase inhibitors, it continues to be an important therapeutic option. Tamoxifen use was attributed to a 40% reduction in recurrent and contralateral disease and can be used as a prophylactic (Cuzick et al. 2007). However, ~40% of women receiving tamoxifen as adjuvant therapy will recur (Early Breast Cancer Trialists’ Collaborative Group 1998) and almost all patients with metastatic disease develop resistance.

The primary mode of action of tamoxifen is by competing with oestrogen for ERα, inhibiting the stimulatory effect of oestrogen for tumour growth. While preclinical evidence for other mechanisms has been produced, there is very little clinical evidence to support these. The benefit from tamoxifen is restricted.
to ER+ tumours (Early Breast Cancer Trialists’ Collaborative Group 1988) other than the small group of ER− progesterone receptor-positive (PgR+) tumours that may be mainly due to false negative ER diagnosis. Our earlier work indicated that approximately one-fifth of recurrent tumours following tamoxifen treatment of ER+ primary disease, showed ER negativity (Johnston et al. 1995, Gutierrez et al. 2005). So while positive ER status is a key determinant of response to tamoxifen, loss of ER can only explain a minority of acquired tamoxifen resistance.

Crosstalk between ER and growth factor receptor pathways has been well documented (Hutcheson et al. 2003, Nicholson et al. 2005). ER can be phosphorylated by MAP kinases downstream of the human epidermal growth factor receptor 2 (HER2) signalling pathway, which can lead to ligand-independent activation (Bunone et al. 1996) while tamoxifen-resistant cells can switch their cell growth dependence between HER2 and ER pathways (Pancholi et al. 2008). In our earlier clinical studies, we found that amplification of HER2 occurred in the tamoxifen-resistant recurrence of 11% of patients who had a HER2− primary (Gutierrez et al. 2005). However, other growth factor receptors have been implicated in the acquisition of tamoxifen resistance including epidermal growth factor (EGFR), HER3 and insulin-like growth factor receptor 1 (IGF1R). In this study, we have focused on aspects of the IGF1R pathway.

IGF1 is a mitogen for human breast cancer cells in vitro, acting primarily via a specific cell surface glycoprotein, IGF1R, especially in the presence of oestriadiol (E2), with which it synergises (Dupont & Le Roith 2001). Breast cancer cell models have shown mixed changes in IGF1R expression with tamoxifen treatment. IGF1R mRNA levels are increased by E2 in MCF7 breast cancer cells (Stewart et al. 1990). No change was observed in MCF7 cells treated with tamoxifen (Guvakova & Surmacz 1997, Chan et al. 2001), but tamoxifen-resistant cells showed reduced IGF1R (Frogne et al. 2005).

IGF binding induces IGF1R autophosphorylation, with p-IGF1R phosphorylating IRS1, IRS2 and Shc, activating transduction pathways such as PI3K and Ras/Raf/MAPK (Riedemann & Macaulay 2006). In addition, IGF1R physically associates with ERz and this leads to activation of IGF1R and the downstream ERK1/2 MAPK pathways. Tamoxifen-resistant MCF7 xenograft models, have higher activated p-IGF1R as opposed to tamoxifen-sensitive models (Massarweh et al. 2008) and clinically, p-IGF1R is associated with poor survival in invasive breast cancer samples (Law et al. 2008).

The PI3K pathway is an important driver of proliferation and cell survival, most notably in cells that are responding to growth factor receptor engagement, such as IGF1R, where binding to ligand causes phosphorylation of PIP2 to PIP3. This recruits PDK1, which phosphorylates and activates Akt, perpetuating downstream signalling. PTEN negatively regulates PI3K by dephosphorylating PIP3. While measurement of each of these components may allow derivation of the individual activities in tamoxifen-resistant breast cancer, stathmin, a regulator of microtubule dynamics, has been identified as a marker of PTEN mutation or loss and PI3K activation in breast cancer (Saal et al. 2007), that acts to integrate the upstream components. Stathmin was found to be an accurate immunohistochemical (IHC) marker of the gene expression signature for IHC-detectable PTEN loss.

Approximately, 80% of PIK3CA mutations occur in three hotspots resulting in single amino acid substitutions; E545K and E542K in the helical domain (exon 9) and H1047R in the kinase domain (exon 20). A recent study of 590 breast cancers showed mutations in one-third of cases (Kalinsky et al. 2009). Mutations were associated with hormone receptor positivity, absence of HER2, low tumour grade and stage and lymph node negativity. An earlier study of 157 hormone receptor-positive tumours suggested that PIK3CA mutations did not have an effect on outcome after tamoxifen therapy (Stemke-Hale et al. 2008).

The primary aim of this study was to determine whether there was evidence for a role of IGF1R and/or the PTEN/PI3K pathway in acquired resistance to tamoxifen by assessing the expression of IGF1R, stathmin and PTEN in paired breast cancer biopsies before and after development of tamoxifen resistance, in addition to the assessment of hotspot mutations in PIK3CA. Any changes in these markers were related to the changes in the expression of steroid receptors and HER2 that we have previously reported in part of this sample set (Johnston et al. 1995, Gutierrez et al. 2005), since the expression of these key parameters markedly affects the interpretation of other changes in phenotype.

**Patients and methods**

**Patients**

Seventy-seven patients diagnosed between 1981 and 2004 at the Royal Marsden Hospital were identified, who had tissue available from both primary invasive tumour and subsequent invasive recurrence following adjuvant tamoxifen treatment. This included 51 patients from an earlier report on steroid receptor and
HER2, but not IGF1R or stathmin (Gutierrez et al. 2005). Previously untreated patients who received tamoxifen for at least 6 months as adjuvant therapy and who had a recurrence at least 6 months after their primary occurrence were classed as tamoxifen-resistant. Patients whose recurrence occurred 6 months after the primary were not included as they were classed as de novo resistant. The time to recurrence was defined as time (in months) between surgery for the primary disease and surgery for the recurrent disease. No patients received neoadjuvant therapy for their primary disease, thus date of surgery was close to the date of presentation. Owing to the era during which patients were treated, there was a group (n = 12) of ER− primaries treated with tamoxifen. These ER− cases were used only to assess the relationship of each marker (PgR, HER2, IGF1R and stathmin) with ER and the main focus was on those 55 paired patients with ER+ primary disease. Menopausal status was not rigorously recorded but 28 of these patients were under the age of 55. Patients were included irrespective of whether they received chemotherapy, or their being on tamoxifen at the time of recurrence. The latter circumstance was considered separately in the data analysis.

**Tissue microarray and immunohistochemistry**

Sections (4 μm) of primary and recurrent tumours were cut and stained by haematoxylin and eosin (H&E) and used to mark areas of invasive tumour for core selection. Tissue microarrays were constructed in triplicate with 0.6 mm cores. Sections (4 μm) were stained for ER, HER2, IGF1R, stathmin and PTEN. In addition, PgR was stained on whole sections, due to the heterogeneous expression of this marker (Quinn et al. 2008). Antigen retrieval for ER and PgR was by microwave for 5 min at full power (900 W) in citrate buffer pH 6.0. Antibody for ER (6F11, Vector Labs, Orton Southgate, Peterborough, UK) was applied at 1/40 dilution and PgR (321, Vector Labs) 1/100. HER2 was detected by HercepTest according to the manufacturer’s instructions. Immunohistochemistry using these antibodies has been widely published (Dowsett et al. 2006, Dowsett et al. 2008). IGF1R (G11, Ventana, Roche Diagnostics, Penzberg, Germany) was stained using the Ventana Benchmark XT at Roche, with antigen retrieval by standard CC1 recovery conditions and 1.7 μg/ml concentration. Validation of this antibody is shown in Supplementary Figure 1, see section on supplementary data given at the end of this article. Stathmin (Cell Signalling, Danvers, MA, USA) was detected following antigen retrieval for 10 min in Target Retrieval Solution pH 9.0 (Dako, Ely, Cambridgeshire, UK) heated at 97 °C by water bath, followed by application of 1/100 antibody and use of this antibody has been previously published (Saal et al. 2007). PTEN was detected using clone 6H2.1 (Dako M3627), with antigen retrieval by microwave in citrate buffer pH 9.0 for 18 min and cooling for 20 min. Visualisation was with Vector ABC. HER2 copy number was determined by fluorescent in situ hybridisation (FISH) with the Pathvysion Her2 DNA probe kit (Vysis, Abbott Molecular, IL, USA).

Nuclear ER and PgR were scored using the histoscore (H-score) and were classed as positive if H-score was >1 (equivalent Allred score ≥3) and >20 respectively. Correlation between two independent scorers for ER and PgR, was very good (Pearson r = 0.93; P = 0.0001 and r = 0.95; P < 0.0001) respectively (n = 10). HER2 was scored positive if the HercepTest was 3+ or 2+ and FISH-positive (ratio of HER2/CEP17 ≥ 2.0). IGF1R was scored by assigning a score for membrane intensity, ranging from 0 to 3 in 0.5 increments. This was a modification of the approach to scoring with the HercepTest, with 0.5 increments of intensity introduced to increase sensitivity and to represent those cases that would otherwise be considered ambiguous. A score of 1.5, for example represents a staining which one might consider a one or two. Weighted Kappa coefficient for IGF1R between two scorers was good (0.65). Cytoplasmic stathmin was described using the modified quick score (Detre et al. 1995) where an overall intensity of 0, 1, 2 or 3 was assigned and multiplied by a factor representing the percentage of cells staining brown (where 0% = 0, 1–4% = 1, 5–19% = 2, 20–39% = 3, 40–59% = 4, 60–79% = 5 and 80–100% = 6). Correlation for this marker was also good between scorers (Pearson r = 0.97; P = 0.001). Nuclear and cytoplasmic PTEN was scored simultaneously by two pathologists, with normal cells taken as an internal control; absence of staining in tumour cells relative to control was scored as zero, staining of a similar intensity to control as one and higher than the control as two (Sakr et al. 2010). Samples were excluded if there were no normal cells present to act as an internal control.

**DNA extraction**

Sections (3 × 8 μm) were microdissected to enrich for tumour content and DNA extracted using a modified version of the DNasey Tissue kit (Qiagen). Briefly, following microdissection, sections were treated overnight with 1 M sodium thiocyanate and washed twice with PBS. Tumour was scraped into 180 μl of buffer...
RLT from the kit and 20 μl proteinase K added, then the manufacturer’s protocol continued. DNA was quantified by PicoGreen (Invitrogen, Paisley, UK).

PIK3CA sequencing

PCR with primers spanning the hotspot mutation regions of exons 9 and 20 PIK3CA gene were performed with 20 ng of DNA (exon 9 F: CTGTAATCCTCGTGAAAT and R: ATTATCGACTTACCTGAGAC and exon 20 F: CAATCTTTTGATGACATTGC and R: TGGAATCCAGAGTGAGCTTT), under the following conditions; 94 °C 10 min (94 °C 30 s, 50 °C 30 s and 72 °C 30 s) × 40, 72 °C 10 min. PCR products were purified and sequenced on an ABI 3130 with the Big Dye 1.1 kit (Applied Biosystems, Warrington, Cheshire, UK) as per manufacturer’s instructions. Derived sequences were blasted against the known gene sequence and any observed mutations confirmed by an independent PCR and sequencing with the reverse primer.

Data analysis

GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used to calculate all statistics. To assess differences between paired primary and recurrence, Wilcoxon matched-pairs test was used. Differences between unpaired groups were measured by the Mann–Whitney U test and Spearman rank was used to determine correlations of datasets. Significance was taken as \( P \leq 0.05 \).

Clustering analyses was performed by data from all biomarkers (ER, PGR, IGF1R, STMN1, HER2, PTEN and PIK3CA). Data for HER2, PTEN and PIK3CA were converted from − and + to binary data (0 and 1 respectively). Missing data has been left as missing. Data for ER, PGR, IGF1R and STMN1 have been normalised so that the scale of these data is from 0 to 1.

Results

Patient demographic

Patient demographics are listed in Table 1. Median patient age was 55 years at diagnosis. A median of 27 months of tamoxifen treatment had been received with a median time to recurrence of 30 months. 11 patients had also chemotherapy between their primary and recurrence date, 14 had radiotherapy and 12 had both chemotherapy and radiotherapy.

Eleven (20%) ER C primary patients were not on tamoxifen at the time of recurrence and had finished endocrine therapy a median of 33 months before (range 17–66 months). These patients had received a median of 60 months tamoxifen and had a median time to recurrence of 90 months. Cases recurring on tamoxifen were therefore also analysed separately, although sample numbers were consequently reduced.

Relationship of biomarkers with ER

Expression of PgR, HER2, IGF1R and stathmin was significantly different between ER+ and ER− primary tumours (Table 2): PgR and IGF1R were higher in ER+ tumours while HER2 and stathmin were lower.

Table 1 Demographics of patients recurring after treatment with adjuvant tamoxifen

<table>
<thead>
<tr>
<th>Feature</th>
<th>All patients (n=77)</th>
<th>ER+ primary paired patients (n=55)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis</td>
<td>55 years (28–81 years)</td>
<td>54 years (28–86 years)</td>
</tr>
<tr>
<td>Duration of tamoxifen</td>
<td>27 months (6–84 months)</td>
<td>36 months (6–84 months)</td>
</tr>
<tr>
<td>Time to recurrence</td>
<td>30 months (6–146 months)</td>
<td>43 months (6–146 months)</td>
</tr>
<tr>
<td>Tumour size</td>
<td>20 mm (3–70 mm)</td>
<td>20 mm (7–70 mm)</td>
</tr>
<tr>
<td>Nodes (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>18 (23%)</td>
<td>13 (24%)</td>
</tr>
<tr>
<td>Negative</td>
<td>15 (20%)</td>
<td>13 (24%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>44 (57%)</td>
<td>29 (52%)</td>
</tr>
<tr>
<td>Grade, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>5 (7%)</td>
<td>3 (5%)</td>
</tr>
<tr>
<td>Grade 2</td>
<td>28 (36%)</td>
<td>21 (38%)</td>
</tr>
<tr>
<td>Grade 3</td>
<td>12 (16%)</td>
<td>9 (16%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>32 (42%)</td>
<td>22 (40%)</td>
</tr>
<tr>
<td>Adjuvant treatment, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>77 (100%)</td>
<td>55 (100%)</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>14 (18%)</td>
<td>11 (20%)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>11 (14%)</td>
<td>8 (15%)</td>
</tr>
<tr>
<td>Radiotherapy and chemotherapy</td>
<td>12 (16%)</td>
<td>4 (7%)</td>
</tr>
</tbody>
</table>

aClinicopathological data were not available for all samples; b\( n = 76 \); c\( n = 50 \); d\( n = 36 \).

Table 2 Biomarker immuno score in primary tumours according to oestrogen receptor (ER) status (median). There was a significant association with ER status with the exception of PTEN. See methods section for scoring methods

<table>
<thead>
<tr>
<th>Marker</th>
<th>Scoring method</th>
<th>Score (n)</th>
<th>Score (n)</th>
<th>Score (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PgR</td>
<td>H-score</td>
<td>105.1 (51)</td>
<td>19.1 (12)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HER2</td>
<td>Positive (%)</td>
<td>13% (49)</td>
<td>57% (14)</td>
<td>0.0002</td>
</tr>
<tr>
<td>IGF1R</td>
<td>Membrane intensity</td>
<td>1.5 (60)</td>
<td>0.8 (16)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Stathmin</td>
<td>Q-score</td>
<td>4.8 (48)</td>
<td>8.1 (10)</td>
<td>0.0075</td>
</tr>
<tr>
<td>PTEN</td>
<td>Positive (%)</td>
<td>73% (40)</td>
<td>75% (12)</td>
<td>0.1349</td>
</tr>
</tbody>
</table>
were lower. This extended to a quantitative relationship between ER and PgR ($r=0.30; P=0.013$), HER2 ($r=0.34; P=0.007$) and stathmin ($r=-0.45; P=0.0003$). However, there was no significant difference in incidence of PTEN positivity between ER+ and ER− tumours.

Considering only ER+ primary tumours, there was no quantitative correlation between ER and PgR, HER2, IGF1R or stathmin (Table 3).

However, there was a significant positive correlation between ER and PgR in the recurrence sample ($r=0.36; P=0.008$; Table 3).

Patients with an ER+ primary tumour had significantly longer TTR than those with an ER− primary (median 41 (range 6–146) months versus 24 (6–82) months; $P=0.030$).

The following data relate to patients with ER+ primary tumours.

**Oestrogen receptor**

Twelve of 55 (22%) ER+ primaries with a recurrent ER measurement available, recurred as ER− disease (Fig. 1a). Distribution of ER H-scores in the primary sample ranged from 5.0 to 275.0, with a median of 95.5. ER− disease occurred only in patients who recurred whilst taking tamoxifen. Nineteen of the 43 patients (44%) with ER+ primary and ER+ recurrent disease had reduced ER by ≥33% (Fig. 1b) at the time of recurrence, seven (16%) an increase of ≥50% (Fig. 1d) and in the remaining 17 (40%) patients any change was within these limits (Fig. 1c). Even in ER+ recurrences, median ER level was significantly lower compared with the same tumours pretreatment (H-score 75.0 (interquartile range (IQR) 17.9–113.0) vs 108.0 (IQR 90.9–130.9) $P=0.005$). However, the reduction of ER only occurred in the recurrence of those on tamoxifen (median H-score 108.2 vs 74.5; $P=0.0003$), not those who recurred after finishing tamoxifen (median H-score 55.2 vs 105.8; $P=ns$).

There was a trend to lower ER levels in the primary tumours that recurred as ER− ($P=0.071$) and these patients had significantly reduced TTR compared with those that recurred as ER+ (median TTR 51.0 vs 19.5 months; $P=0.002$).

However, there was no significant difference of PgR, HER2, IGF1R or stathmin in the primary tumour of patients according to the ER status of the recurrent lesion (Table 4).

**Progesterone receptor**

Eleven patients (22%) had an ER+ primary that was PgR−. Levels of PgR in primary tumours that recurred as ER− were lower than those primary tumours that recurred as ER+ (Table 4) and had a shorter TTR (30 vs 42.5 months), but these differences were not significant. Overall, there was a significant reduction of PgR in recurrent tumours (median H-score pre- 122.4 versus post- 21.8; $P<0.0001$). This was also the case separately in those that remained ER+ (median H-score pre- 132.3 versus post- 32.3; $P=0.004$) and those which recurred as ER− (median H-score pre- 71.0 versus post- 8.9; $P=0.001$).

There appeared to be a greater reduction of PgR in tumours that had an ER− (Fig. 2a) or ER low (H-score 1–25; Fig. 2b) recurrence than an ER moderate to high

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**Table 3** Correlation of markers with oestrogen receptor (ER) in ER+ primary tumours and their matched recurrence. The only significant association with ER is progesterone receptor (PgR) in the recurrence sample.

<table>
<thead>
<tr>
<th>Marker (n)</th>
<th>Primary Spearman r</th>
<th>P value</th>
<th>Recurrence Spearman r</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PgR (54)</td>
<td>−0.12</td>
<td>0.382</td>
<td>0.36</td>
<td>0.008</td>
</tr>
<tr>
<td>HER2 (48)</td>
<td>−0.18</td>
<td>0.213</td>
<td>0.08</td>
<td>0.598</td>
</tr>
<tr>
<td>IGF1R (49)</td>
<td>0.00</td>
<td>1.000</td>
<td>0.24</td>
<td>0.103</td>
</tr>
<tr>
<td>Stathmin (49)</td>
<td>−0.17</td>
<td>0.254</td>
<td>0.13</td>
<td>0.391</td>
</tr>
</tbody>
</table>

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**Figure 1** ER expression in primary and tamoxifen-resistant recurrences. ER expression was lost completely in 22% of recurrences (a). In ER-positive recurrences, ER levels were reduced by more than one-third in 44% of recurrences (b) and increased by more than half in just seven recurrences (c) ER expression remained relatively consistent (d) in the remaining tumours which had originally presented as ER+. Red lines indicate those patients whose recurrence occurred after completing tamoxifen therapy.
recurrences (Fig. 2c). PgR status changed in four patients from negative to positive, and this was restricted to tumours with an ER H-score ≥ 25 at recurrence (Fig. 2c). Patients with an ER+PgR+ primary tumour that recurred as PgR− had longer TTR than those whose tumour recurred as PgR− (49 vs 30 months), but this was not statistically significant.

In those patients recurring whilst taking tamoxifen (n = 43), 28% had an ER+ primary that was PgR−. Overall, there was a significant reduction of PgR in recurrent tumours (P = 0.0002). This was also the case separately in those that remained ER+ (P = 0.014) and those which recurred as ER− (P = 0.001).

Table 4 Biomarker immuno score in oestrogen receptor-positive (ER+) primary tumours, according to their recurrent ER status (median)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Scoring method</th>
<th>1+ of ER+ recurrence</th>
<th>1+ of ER− recurrence</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>H-score</td>
<td>108.0 (43)</td>
<td>79.7 (12)</td>
<td>0.071</td>
</tr>
<tr>
<td>PgR</td>
<td>H-score</td>
<td>132.3 (39)</td>
<td>71.0 (12)</td>
<td>0.418</td>
</tr>
<tr>
<td>HER2</td>
<td>Positive (%)</td>
<td>11% (36)</td>
<td>10% (10)</td>
<td>1.000</td>
</tr>
<tr>
<td>IGF1R</td>
<td>Membrane intensity</td>
<td>1.51 (37)</td>
<td>1.75 (12)</td>
<td>1.000</td>
</tr>
<tr>
<td>Stathmin</td>
<td>Q-score</td>
<td>2.5 (37)</td>
<td>5.6 (12)</td>
<td>0.121</td>
</tr>
<tr>
<td>PTEN</td>
<td>Positive (%)</td>
<td>74% (34)</td>
<td>64% (11)</td>
<td>0.718</td>
</tr>
</tbody>
</table>

Figure 2 PgR, IGF1R and stathmin expression alterations in tamoxifen-resistant recurrences, based on recurrent ER level. Panels a–c show change of PgR according to recurrent ER level, with red lines indicating the only four patients in the cohort that switched from PgR− to PgR, these were confined to the patients with high levels of ER at recurrence. Panels d–f show change of IGF1R. Panels g–i show change of stathmin; 42% of tumours with an ER− recurrence showed an increase of stathmin (g). This was compared with 45% of tumours with a low (h) and 73% of tumours with a high level of ER at recurrence with an increase of stathmin (i).
Human epidermal growth factor receptor 2

HER2 data for paired primary and recurrent tumours was available for 50 ER+ primary cases. HER2 positivity occurred at similar frequency (~10%) in ER+ primaries that recurred as ER+ or ER− (Table 4). Of all ER+ primaries, 42 (84%) were HER2− and remained HER2− in the recurrence. Four cases were HER2+ and remained so. One case was HER2+ in the primary setting and HER2− at time of recurrence. Only three (6%) patients acquired HER2 positivity (as previously reported in the smaller sample set (Gutierrez et al. 2005)); this was associated with loss of ER in one case. When HercepTest scores were assessed, 11 cases showed some increase of HER2 between primary and recurrence (e.g. 0–1 and 1–2), including those three who were FISH confirmed to change from HER2− to HER2+. Of these 11, seven (64%) showed some loss of ER (Fig. 3). All of the cases that recurred after finishing tamoxifen were HER2− in both the primary and recurrent tumour.

Insulin-like growth factor receptor 1

IGF1R data for paired primary and recurrent tumours was available for 49 cases. There was no difference in primary IGF1R expression in tumours that went on to recur as ER+ or ER− (Table 4). There was a significant reduction of IGF1R in recurrent tumours (P<0.0001). This was true in both the group that retained ER+ status (P<0.0001) and that were ER− at the time of recurrence (P=0.004). Approximately, 75% of tumours that recurred as ER+ showed a reduction of IGF1R. A case losing IGF1R at recurrence is shown in Fig. 4a and b. Median reduction in IGF1R expression was similar in ER− (Fig. 2d) and ER+ (both low and high ER; Fig. 2e and f) recurrences.

Figure 3 Change of ER in cases with increased HER2 at the time of recurrence. There was a loss of ER in 64% of cases that had some gain of HER2.

Figure 4 Immunohistochemistry of membrane IGF1R and cytoplasmic stathmin in an ER+ primary tumour and the ER+ recurrence following tamoxifen treatment. Clear IGF1R membrane staining is observed in the primary (a), which is reduced following tamoxifen treatment and the development of resistance (b). In the same patient, cytoplasmic stathmin staining is absent in the primary (c) and is higher in the recurrence (d), suggesting activation of the PI3K pathway.
The lack of a significant quantitative relationship between IGF1R and ER levels pretreatment persisted in the recurrence. There was, however, a weak relationship between the differences in ER and IGFIR level between primary and recurrence ($r=0.34; P=0.018$).

In 39 patients recurring on tamoxifen, IGF1R data for paired primary and recurrent tumours was available. IGF1R expression in primary tumours did not differ significantly between those that went on to recur as ER+ compared with those that recurred as ER−. As in the complete patient set, there was a significant reduction of IGF1R in the recurrent tumours ($P<0.0001$). This was true in both the group that retained ER+ status ($P<0.0001$) and that were ER− at the time of recurrence ($P=0.004$). Approximately, 85% of tumours that recurred as ER+ showed a reduction of IGF1R.

### Stathmin

Stathmin levels were higher in primary tumours that recurred as ER− than ER+ (Table 4), although this was not significant. The majority of (59%) ER+ primary cases showed an increase of stathmin at the time of recurrence ($P<0.005$; example Fig. 4c and d), with both ER+ and ER− recurrences showing an increase (Fig. 2g−i), although only significantly so in the subgroup of patients with maintained ER positivity ($P=0.004$). The greatest proportion of patients (73%) with increased stathmin was observed in those that had high ER (H-score ≥25) recurrent disease (Fig. 2i) compared with those with ER− or ER low recurrences (42 and 45%, respectively; Fig. 2g and h). Cases with increased levels of stathmin at recurrence compared with primary, had a non-significant trend to longer TTR (median 49.0 vs 28.5 months; $P=0.086$).

Changes in stathmin and ER were not quantitatively related. There was no correlation between levels of stathmin and IGF1R in primaries, or in their difference of expression, but there was a trend to a weak positive relationship in recurrences ($r=0.28; P=0.056$).

Changes were similar in just those patients who recurred whilst taking tamoxifen. Stathmin levels were higher in primary tumours that recurred as ER− than ER+ ($P=0.054$) and the majority of (55%) ER+ primary cases showed an increase of stathmin at the time of recurrence. The greatest proportion of patients (76%) with increased stathmin was observed in those with high ER (H-score ≥25) compared with ER− or low recurrences (42 and 33% respectively). Changes in stathmin and ER were not quantitatively related. There was no correlation between levels of stathmin and IGF1R in primaries, or in their difference of expression.

### PTEN

Tumours that remained ER+ at recurrence ($n=34$), showed maintained PTEN expression in 68% and only 6% lost PTEN at the time of recurrence. 18% were PTEN− in the primary and recurrent tumour and a further 9% acquired PTEN positivity. This was in contrast to those who became ER− ($n=11$), where 27% also showed loss of PTEN (Fig. 5). Overall, absence of PTEN occurred in the primary tumour in 27% of those who retained ER positivity and 36% who lost ER.

There was no difference in levels of stathmin between PTEN+ and PTEN− tumours in either primary or recurrent tumours.

### PIK3CA hotspot mutations

Twenty-one pairs of tumours, including ER− primary samples, were sequenced for three hotspot mutations, in exons 9 and 20. A mutation was identified in five
cases and in all five the mutation occurred in both the primary and recurrent sample. The E545K mutation in exon 9 was observed in one patient with an ER+ to ER− resistant tumour, the H1047R mutation in exon 20 in two ER+ to ER+ tumours and one ER− to ER− tumour and the H1047L mutation in exon 20 was observed in one tumour that acquired ER positivity. Overall, the hotspot mutation rate was 3/14 of ER+ tumours, or 21%.

Summary

Many changes in biomarker expression occur in the acquisition of tamoxifen resistance. To achieve an overview of the data we conducted hierarchical clustering analyses and created heatmaps based on the pretreatment expression of the seven parameters (Fig. 6a). This clustering is then applied to the recurrent samples and the differences between the pretreatment and recurrent samples in Fig. 6b and c. HER2 and PTEN status appear to dominate the separation of tumours before treatment, with there being near absolute separation of PTEN-positive from negative tumours. TheHER2-positive tumours cluster together within the PTEN-positive and PTEN-negative clusters. However, the pretreatment clustering does not impose a clear clustering of the recurrent phenotypes.

Discussion

Resistance to tamoxifen occurs clinically in ~40% of adjuvant treated patients and almost all metastatic patients. Despite the increasing use of aromatase inhibitors, tamoxifen remains an important therapeutic option, both for pre-menopausal women and as a sequential regimen with aromatase inhibitors. There have been relatively few clinical reports of changes occurring in the acquisition of tamoxifen resistance; here, we describe a set of matched primary and tamoxifen-resistant recurrences. Differences in expression of the biomarkers are substantial but complex. A number of changes occur that are consistent with concepts of tamoxifen resistance, but these vary greatly between patients. The clustering based on pretreatment samples showed dominance

Figure 6 Heatmaps of 55 ER+ patients with excision biopsies pre- and post-tamoxifen therapy. (a) Primary tumours were clustered based on the expression values of the seven biomarkers (scaled data). Only black and red colours are observed, as the data has not been centred, i.e. this heatmap represents absolute expression values after data scaling as described, where black is low expression, red high and grey indicates missing data. (b) Scaled data for the recurrent tumour is shown below the corresponding primary; (c) the difference between primary and recurrent expression is shown (scaled data), with green showing reduced expression, red increased expression, grey is missing data and black is no change.
of PTEN and HER2 status in this marker set but it is possible that this largely reflects the dichotomous nature of the data on these two markers. The strong pretreatment patterns were not persistent through to the recurrent lesions indicating that pretreatment phenotype does not strongly predict for on phenotype at recurrence.

ER levels were significantly reduced, and sometimes lost altogether in tamoxifen-resistant recurrences. While reduced PgR might be expected following tamoxifen treatment because of its antioestrogenic activity, there is little evidence that this occurs. Rather, presumably due to a substantial agonist effect of tamoxifen on this gene’s expression, PgR expression is increased in many ER+ breast tumours shortly after starting tamoxifen treatment (Howell et al. 1987). Also, in patients treated with tamoxifen as primary medical therapy who acquired resistance, there was little loss of PgR (Johnston et al. 1995). The suppression of PgR confirmed in this study and previously reported in the adjuvant tamoxifen-resistant setting (Johnston et al. 1995), may therefore be explained by loss of ER functionality. This is supported by the greater loss of PgR shown in the tumours with low or negative ER recurrences (Fig. 2), as well as the suppression of oestrogen-regulated IGF1R.

In our previous report (Gutierrez et al. 2005), we showed a positive association of ER status with PgR in the primary setting, which was lost at recurrence and this was confirmed here. In this updated set, looking at only ER+ primary tumours, no quantitative relationship with PgR was observed in primary tumours, but was present at resistance, probably due largely to the ER− recurrences, again supporting the above hypothesis.

Only 10% of ER+ tamoxifen-resistant patients were HER2+ initially and only 6% acquired HER2 at the time of recurrence. No tumours were found to acquire HER2 positivity in the recurrence beyond the three cases previously reported (Gutierrez et al. 2005), confirming that absolute gain of HER2 is an uncommon occurrence. However, a further eight cases showed some increase of HER2 protein. ER was reduced in the majority of these cases (64%).

The data presented here show for the first time that IGF1R is reduced in the recurrence of many tamoxifen-resistant patients. It has been reported that tumours showing primary resistance to tamoxifen are frequently IGF1R poor while those that respond, IGF1R rich (Gee et al. 2005). Tamoxifen-resistant cells also have a down-regulation of IGF1R compared with parental cells, although phosphorylated levels remain the same (Knowlden et al. 2005). The suppression of IGF1R therefore appears to be a consistent feature of the tamoxifen-resistant phenotype. Whilst the loss of IGF1R was weakly associated with loss of ER, there is evidence that this is an independent event as even tumours that showed no significant loss of ER, had a significant loss of IGF1R. A positive association of IGF1R has been observed previously with ER and PgR (Pekonen et al. 1988, Peyrat et al. 1988, Gee et al. 2005); however, others (including ourselves in this series) have not observed the relationship with PgR (Papa et al. 1993). Time to progression was significantly increased for IGF1R rich patients and there was longer TTR for patients whose recurrence showed an increase of IGF1R. The data indicating lower expression of IGF1R in tamoxifen resistance are relevant to the targeting of the large number of IGF1R kinase inhibitor and antibodies in clinical development.

Stathmin has been identified as an immunohistochemical marker of activation of the PI3K pathway and lack of PTEN (Saal et al. 2007). However, the relationship of stathmin with PTEN expression was not observed in this cohort, with no difference in stathmin levels between PTEN+ and PTEN− tumours, suggesting that taken alone, stathmin may not be a good marker of PTEN. No relationship between ER and stathmin in ER+ primary cases was observed in this study confirming previous reports (Golouh et al. 2008). A negative relationship between ER status and stathmin was observed consistent with another study, including cell lines (Brattsand 2000). A negative relationship with ER has also been shown to hold at the stathmin mRNA level (Curmi et al. 2000).

A significant increase in stathmin expression occurred in the tamoxifen-resistant recurrence of ER+ primary tumours, suggesting activation of the PI3K pathway. Although stathmin levels are higher in ER− primary tumours, interestingly there was no significant increase of stathmin in patients developing an ER− recurrence, but this was a small subgroup in our study. Furthermore, in primary tumours there was no relationship of stathmin with IGF1R, despite this putative common association with an activated PI3K pathway. A positive association was observed at recurrence, suggesting that some coalescence of their activities may be acquired. Golouh et al. (2008) reported that high stathmin staining intensity in the primary tumour was associated with shorter disease-free survival in tamoxifen-treated ER+ patients. Stathmin has also been found to be overexpressed in the presence of mutant Tp53 (Alli et al. 2007), a feature associated with poor response to tamoxifen (Berns et al. 2000). Stathmin overexpression has been associated with reduced sensitivity to
chemotherapeutic drugs (Alli et al., 2002). Despite a number of patients having received chemotherapy in addition to tamoxifen in this study, none were treated with a regimen that targeted microtubules.

Further evidence consistent with PI3K activation with acquisition of tamoxifen resistance comes from lower PgR levels in tamoxifen-resistant recurrences. IGF1 mediated decreases of PgR protein and mRNA levels via an inhibition of transcription have been demonstrated in breast cancer (Cui et al., 2003), with the PI3K/Akt/mTOR pathway being specifically implicated. The reduction of PgR levels in this series was found to be independent of ER activity. Furthermore, PTEN loss (implicated in this study by increased stathmin) and reduced PgR have been observed both in vitro (Miller et al. 2009) and in vivo (Saal et al. 2005). However, our data, although limited in number, suggest that PTEN loss contributes to only a small proportion of tamoxifen-resistant tumours, with the majority of ER+ recurrent tumours maintaining PTEN positivity. Less than a quarter of patients that recurred on tamoxifen showed a PIK3CA mutation in the primary tumour; we found no evidence of this frequency increasing at recurrence.

Immunohistochemistry is a useful tool to assess these markers, particularly in the tissue microarray setting as many samples can be stained at once. However, not all markers can be well represented in a tissue microarray (TMA) format and for this reason the heterogeneous staining PgR was performed on whole sections in this study. The use of ER, PgR and HER2 is well documented and while the research community is increasingly becoming aware of the instability of certain markers in stored fixed biopsies over time, we can be reassured in this study by the fact that most markers showed reduced levels in the recurrent (more recent) sample.

In summary, we have confirmed the loss of ER and PgR in tamoxifen-resistant recurrences and shown that acquisition of HER2 positivity is an uncommon event. There is no evidence for increases in IGF1R or in mutations in PIK3CA, but increases in stathmin levels, and infrequent loss of PTEN indicate activation of the PI3K pathway in some tamoxifen-resistant breast tumours. It is unlikely that a single mechanism of resistance exists; molecular characterisation of individual recurrent lesions is needed to select the most appropriate treatment in progressive advanced disease.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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