Measuring IGF-1, ER-α and EGFR Expression Can Predict Tamoxifen-resistance in ER-positive Breast Cancer

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Abstract. *In vitro studies have suggested that tamoxifen resistance may be due to altered expression and downstream signalling of insulin-like growth factor-1 (IGF-1) receptor (IGF-1l), oestrogen receptor-alpha (ERα), epidermal growth factor receptor (EGFR) and HER-2. We investigated which gene expressions could predict tamoxifen resistant breast cancer. Expression of IGF-1R, IGF-1 ligand (IGF-1), ERα, EGFR and HER-2 in 91 ER-positive breast cancer tumours were measured using real-time PCR and correlated with clinical outcome. The tamoxifen resistant group (n=20) consisted of: i) tumours which were resistant to neoadjuvant tamoxifen treatment and ii) tumours which were excised from patients who later developed recurrence or metastasis during adjuvant tamoxifen treatment. These were compared with tamoxifen sensitive tumours which were surgical excision specimens from patients who did not develop recurrence/ metastasis during adjuvant tamoxifen treatment. Tumours with higher IGF-1 ligand and ERα expression took longer to develop tamoxifen resistance. Tamoxifen resistant tumours had lower IGF-1 and ERα expression compared to tamoxifen-sensitive tumours. IGF-1 expression strongly correlated with ERα expression in the tamoxifen sensitive group only. ERα inversely correlated with EGFR expression in the tamoxifen resistant group only. We conclude that IGF-1 ligand and ERα expression in breast carcinomas can be measured to predict tamoxifen resistance. Measuring ERα expression using RT-PCR may be more sensitive than immunohistochemistry in determining anti-oestrogen sensitivity.*

Anti-oestrogen resistance is a major challenge in the treatment of oestrogen receptor alpha (*ERα*)-positive breast cancer patients. Despite being the 'gold-standard' for the hormonal treatment of *ERα*-positive breast cancer (27), tamoxifen is still not effective in up to 30% of immunohistochemically (IHC) *ERα*-positive breast tumours (10). It has been suggested that the unpredictability of therapeutic responses may lie in the current *ERα* assay which utilises immunohistochemistry and Allred scoring (10). However, extensive research has revealed that the tamoxifen resistant breast cancer phenotype may also be due to altered cellular downstream pathways such as those involving the insulin-like growth factor-1 receptor (*IGF-1R*), oestrogen receptor-alpha (ERα), epidermal growth factor receptor (*EGFR*) and *HER-2* (20, 21, 28). Reports on whether *IGF-1R* is elevated or decreased in anti-oestrogen-resistant breast cancer cell lines are conflicting. Some studies suggest that *IGF-1R* expression and activity are important in maintaining a tamoxifen resistant phenotype (20, 23) while another study showed that a fall in *IGF-1R* expression was seen tamoxifen resistant cell lines (3). The down-regulation of *ERα* expression in breast cancer cells has also been suggested as a feature of tamoxifen resistance (25). Several studies have suggested that overexpression of *EGFR* and *HER-2* in *ERα*-positive breast cancer cells can lead to resistance to anti-oestrogens (16, 19).

The purpose of this study is to explore the pattern of gene expression in the tumour tissue of tamoxifen resistant and sensitive breast cancers. Firstly, we aimed to see whether *IGF-1, IGF-1R, ERα, EGFR* or *HER-2* mRNA levels could be used to predict shorter time-to-failure of tamoxifen treatment. Next, we studied whether *IGF-1, IGF-1R, ERα, EGFR* and *HER-2* mRNA expression levels were different in tamoxifen resistant breast cancers compared to tamoxifen sensitive breast cancers. Finally, we also looked at correlations between *IGF-1, IGF-1R, EGFR, HER-2* and *ERα* mRNA levels in tamoxifen resistant and tamoxifen sensitive breast cancers to see if any gene expression patterns could be associated to any one phenotypes.

Patients and Methods

Patient population. These consisted of patients who underwent breast surgery for breast cancer, as well as adjuvant therapy, at St George's Hospital, London from September 1997 to September 2002. All patients were confirmed to be free of distant metastasis pre-operatively. Post-operatively, most of these patients received

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adjuvant medical treatment for breast cancer for improvement of prognosis. Only patients whose breast tumours were *ERα*-positive on histopathology were recruited for this study. They were subsequently treated with tamoxifen for 5 years. All patients who underwent breast conservation surgery were given radiotherapy. Depending on the grade and stage of the breast cancer, patients were selectively given chemotherapy. After completing both surgical and adjuvant treatment, patients were followed up annually at the Outpatient Clinic. During follow-up, patients who developed recurrence or metastasis while on adjuvant tamoxifen were identified. Time-to failure of tamoxifen treatment was considered to be the time from commencement of tamoxifen treatment to the time of discovery of recurrent or metastatic breast cancer (in months).

Patient selection. Only *ERα*-positive breast cancer specimens from patients were used for this study. The series was divided into two broad groups: tamoxifen resistant (Tam-R) group and tamoxifen sensitive group. The clinical criterias of each group were as follows: Tamoxifen resistant (Tam-R) group (n=23). This group consisted of two subgroups:

i) Tam-R1 subgroup:
cases which failed to respond to primary tamoxifen treatment (n=11). This group consisted of breast cancer cases which were confirmed as being tamoxifen resistant based on clinical assessment before surgical excision. These were a small number of patients who were diagnosed to have *ERα*-positive breast cancer on core biopsy histology. However, due to their advanced age, significant medical co-morbidities such as significant cardiovascular disease, chronic obstructive pulmonary disease, general frailty or personal preference, they did not initially undergo surgery. Instead, they were treated with primary tamoxifen treatment. On routine follow-up, some of these tumours continued to progress (reflected by increase in size on serial examination/ measurements) despite being treated with tamoxifen and were considered *de novo* resistant tumours. If the tumour initially responded to tamoxifen treatment (as reflected by a reduction or no change in tumour size on serial examination/ measurements) for at least a year but started to increase in size again after that, these tumours were considered acquired resistant tumours. Both of these types of cancer were considered as definite tamoxifen-resistant breast cancer. Because these breast tumours progressed while on primary tamoxifen treatment, these patients eventually underwent surgical excision (mastectomy or breast conserving surgery). Tissue samples were obtained from these surgical excision specimens.

ii) Tam-R2 group:
cases which developed recurrence/metastasis while on adjuvant tamoxifen treatment (n=12). This group consisted of a small number of patients who were treated by mastectomy/breast conserving surgery soon after being diagnosed with breast cancer. As their histology showed *ERα*-positivity, these patients were started on adjuvant tamoxifen of 20 mg per day soon after completing their surgery and adjuvant treatment (in the form of chemotherapy and/or radiotherapy to the breast). However, within 5 years of the primary treatment, these patients developed breast cancer recurrence or metastasis. Based on this clinical pattern, their initial tumours could have been either tamoxifen resistant before surgery (*de novo* resistance) or were initially tamoxifen sensitive but residual or disseminated breast cancer cells later develop into a tamoxifen resistant phenotype during tamoxifen treatment (acquired resistance). Tissue samples were obtained from these surgical excision specimens (before starting tamoxifen).

Tamoxifen-sensitive group (n=69). This group consisted of a large number of patients who underwent a mastectomy or breast-conserving surgery soon after being diagnosed with breast cancer and were administered appropriate adjuvant treatment (chemotherapy and/or breast radiotherapy). As their tumours were histologically *ERα*-positive, these patients were started on adjuvant tamoxifen 20 mg per day soon after surgery. These patients did not develop any breast cancer recurrence or metastasis during the 5 years of follow-up. Based on this clinical pattern, these breast cancer cases were considered to be tamoxifen sensitive cases.

Breast tissue collection, storage, homogenisation, RNA extraction, cDNA preparation and real-time (Q-PCR). Institutional guidelines involving ethical approval and obtaining patient informed consent were followed. Immediately after surgical excision, the tumour tissue was excised from the main breast specimen and stored in a tissue bank at a temperature of –140˚C until the commencement of this study. For this study, approximately 10 mg of tumour tissue were homogenized. The concentration of RNA was determined using a UV spectrophotometer (Wolf Laboratories, York, UK) to ensure adequate amounts of RNA for analysis. Reverse transcription was carried out using a reverse transcription kit (AbGene, Kent, UK) with an anchored olig (dT) primer using 1 μg of total RNA in a 96-well plate to produce cDNA. Conditions for reverse transcriptase were 25˚C for 10 minutes, 48˚C for 30 minutes and 95˚C for 5 minutes. The level of these transcripts from the above prepared cDNA was determined using real-time Q-PCR based on the Amplifluor technology modified from a method reported previously (11). PCR primers were designed using Beacon Designer software but to the reverse primer had an additional sequence, known as the Z sequence (5'-actgaacctgaccgtaca-3') which is complementary to the universal Z probe (Intergen Inc., Oxford, UK). The region of amplification expanded over at least one intron. The forward and reverse primer sequences are listed in Table I. The probes were intron spanning to prevent amplification of genomic DNA. The reaction was carried out using the following: Hot-start Q-master mix (Abgene), 10 pmol of specific forward primer, 1 pmol reverse primer with the Z sequence, 10 pmol of FAM-tagged probe (Intergen Inc.) and cDNA from 50 ng of RNA. The reaction was carried out in the I-cyclerIQ machine (BioRad, Hemmel Hemstead, UK) equipped with an optic unit that allows real-time detection of 96 reactions under the following conditions: 94˚C for 12 min and 50 cycles of 94˚C for 15 s, 55˚C for 40 s and 72˚C for 20 s. The results of the test molecules were normalized against levels of β-actin (internal standard) using a β-actin quantitation kit (Perkin-Elmer, Cambridge, UK) that was simultaneously amplified with the samples in the 96-well plate.

Statistical analysis. All statistical analyses were carried out using SPSS (Version 14.0) computer software (SPSS Inc., Middlesex, UK). When analyzing time-to-failure of tamoxifen treatment, mRNA readings were divided into two groups using the median of the mRNA readings as a cut-off point. This method was adopted in a previous similar study (24). Curves were analysed using the Kaplan-Meier method and comparison between curves was evaluated using the log-rank (Mantel-Cox) test. Differences in gene expression between groups were analysed using the non-parametric Mann-Whitney test. Correlations between mRNA levels were carried out using non-parametric Spearman's log-ranked correlation test. Statistical significance was determined if the p-value was <0.05.

Table I. *Sequence of primers used in real-time Q-PCR.*

Gene	Forward primer (F1)	Reverse primer (Zr)
IGF-1	5'-agtgctgtatgcctctgtg-3'	5'-actgaactgaccgtacaggctcatggtgatcttctc-3'
IGF-1 R	5'-agtctcttcagttcgtgtgt-3'	5'-actgaacctgaccgtacagaagcagcactcatccac -3'
ERα	5'-cctactacctggagaacgag-3'	5'-actgaactcttcggtcttttcgtatg-3'
EGFR	5'-gacctccatgcctttgagaa-3'	5'-actgaacctgaccgtacagcacaaatttttgtttcctga-3'
HER-2	5'-gtggacctggatgacaag-3'	5'-actgaacctgaccgtacagaccacgaccagcagaat-3'
β-actin	5'-atgatatcgccgcgctcgtc-3'	5'- cgctcggtgaggatcttca-3'

Table II. *Demographics, histology and follow-up details of the study population.*

Demographics and histology	Tam-R1 group	Tam-R2 group	Tamoxifen-sensitive group
No of cases, n	11	12	69
Age (years) (mean/median/range)	72/74/ 54-87	60.3/57.0/34-85	61.2/61.0/33-89
Grade			
1	0	0	17
2	4	3	31
3	7	8	21
Lymph node status			
Positive	7	9	26
Negative	2	1	36
Not assessed	2	2	7
Size of tumour			
<2 cm	5	4	32
2-5 cm	3	7	29
>5 cm	3	0	6
Not available	1		2
ER immunoscoring			
3-4	0	1	8
5-6	2	2	6
7-8	9	9	55
Time to tamoxifen failure (months) (mean/median/range)	49.4/49.0/3-89	34.6/33.0/12-55	None
Tamoxifen resistant status			
De novo	2	0	Not applicable
Acquired	11	12	

Results

Patient demographics and histology. There were a total of 92 *ERα*-positive patients included in this study. The demographics of the patients and histological details of their breast cancers are described in Table II.

IGF-1, IGF-1R, ERα, EGFR and HER-2 expression in breast cancer specimens as a predictor of time-to-failure of Tamoxifen treatment. Only patients in the Tam-R2 (n=12) and tamoxifen sensitive group were included into the Kaplan-Meier test (n=81). Patients from the Tam-R1 group were excluded as these were already known to be tamoxifen resistant at the time of initial surgery. Higher *IGF-1* expression in these patients was significantly associated with longer time-to-failure of tamoxifen treatment ($p<0.001$) (Figure 1). *IGF-1R* expression in *ERα*-positive tumour tissue was not statistically associated with time-to-failure of tamoxifen treatment ($p=0.476$) (Figure 2). The *EGFR* and *HER-2* mRNA expression levels in tumour tissue were not statistically associated with time-to-failure of tamoxifen treatment ($p=0.205$ and 0.102 respectively).

We found that tumours with higher *ERα* mRNA levels had longer time-to-failure of tamoxifen treatment compared to tumours with lower *ERα* expression ($p=0.013$) (Figure 3). Using IHC and the Allred scoring system, we tested whether *ERα* expression could predict time-to failure of tamoxifen treatment; we divided our series into those with immunoscore of 3-4 *vs*. 7-8. We did not find any associations between ERα

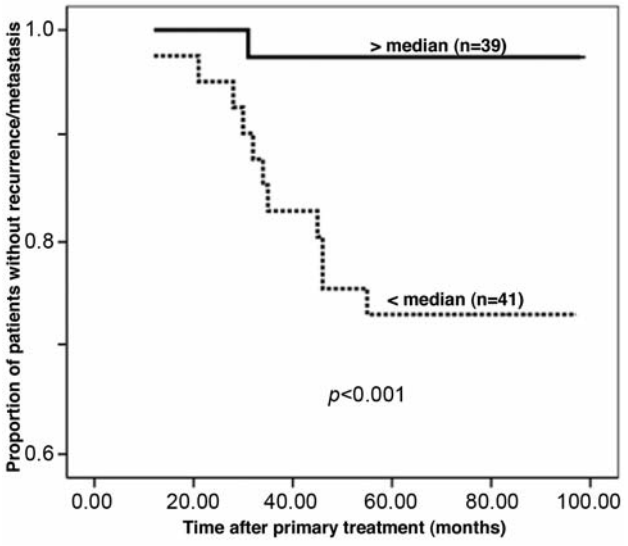

Figure 1. *Relationship between IGF-1 mRNA expression (above and below median) and time-to-failure of tamoxifen treatment.*

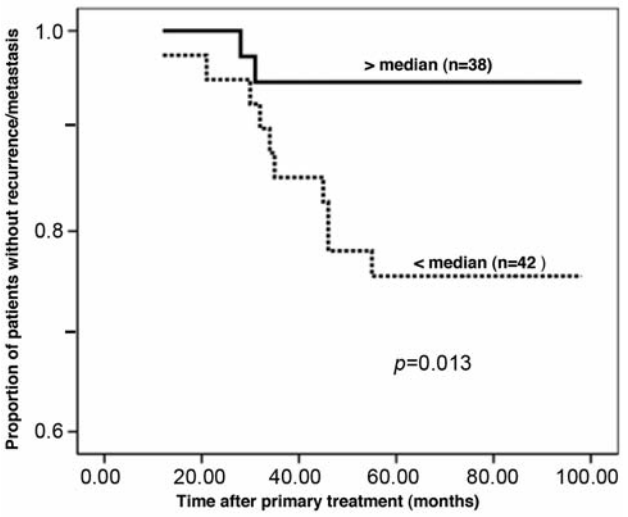

Figure 3. *Relationship between ERα mRNA expression (above and below the median) and time-to-failure of tamoxifen treatment.*

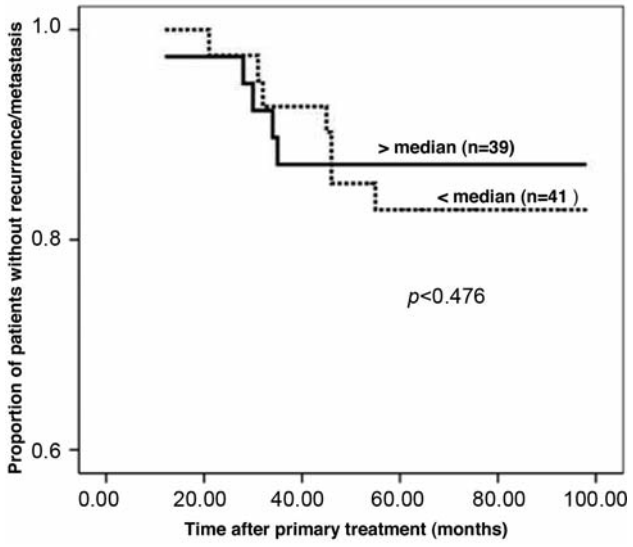

Figure 2. *Relationship between IGF-1R mRNA expression (above and below the median) and time-to-failure of tamoxifen treatment.*

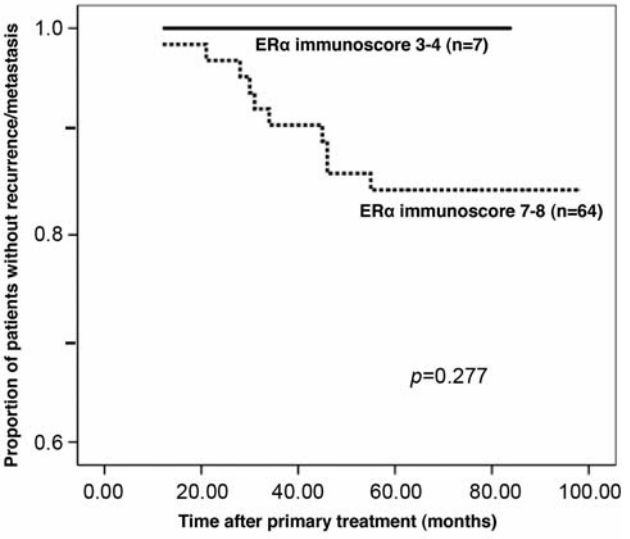

Figure 4. *Relationship between ERα immunoscoring and time-to-failure of tamoxifen treatment.*

immunoscoring and time-to-failure of tamoxifen treatment (p=0.277) (Figure 4).

Using Cox multivariate analysis, *IGF-1* mRNA levels, histopathological grading and lymph node (LN) status were independent predictors of time-to-failure of tamoxifen treatment. The results of uni- and multivariate analyses of the relationship between gene expression, histology factors and time-to-failure of tamoxifen treatment are presented in Table III.

Expression of IGF-1, IGF-1R, ERα, EGFR and HER-2 in the tamoxifen resistant and tamoxifen sensitive groups. We did not find any difference in *IGF-1, IGF-1R, ERα, EGFR* and *HER-2* mRNA levels between the Tam-R1 and Tam-R2 groups (Table IV). There was significantly lower expression of *IGF-1* and ERα mRNA levels in the tamoxifen resistant group (Tam-R1 and Tam-R2 together) compared to the tamoxifen sensitive group (p=0.002 and 0.026 respectively) (Table V). When analysed separately, *IGF-1* mRNA levels

Table III. *Cox uni- and multivariate analyses of the relationship between gene expression/histopathology factors and time-to-failure of tamoxifen treatment.*

Gene expression (mRNA level)		Univariate analysis				Multivariate analysis	
		No. of patients	No. of events	Log-rank *p*-value	Hazard ratio (95% CI)	*p*-value	Hazard ratio (95% CI)
IGF-1	<median	41	12	0.001	0.12 (0.02-0.91)	0.004	0.253 (0.098-0.652)
	>median	39	1				
IGF-1R	<median	41	7	0.476	0.94 (0.32-2.79)	0.570	
	>median	39	6				
ERα	<median	42	11	0.013	0.15 (0.03-0.67)	0.617	
	>median	38	2				
EGFR	<median	39	4	0.205	2.19 (0.66-7.28)	0.570	
	>median	36	8				
HER-2	<median	41	6	0.102	0.29 (0.0795.79)	0.162	
	>median	39	5				
Grade	1	12	0				
	2	24	3	0.023	4.41 (1.31 -14.87)	0.004	7.02 (1.889-26.10)
	3	38	8				
Lymph node status	Positive	35	9	0.005	10.72 (1.36 -84.65)	0.015	13.36 (1.67-106.93)
	Negative	37	1				
Tumour size	<2.0 cm	36	4				
	2.0-5.0 cm	35	7	0.329	1.08 (0.43-2.70)	0.558	
	>5.0 cm	6	0				

Table IV. *Significance of differences in gene expression between Tam-R1 and Tam-R2 breast cancer.*

Gene expression	Tam-R1 group (n=11)	Tam-R2 group (n=12)	*p*-Value
IGF-1R	13.45	10.67	0.325
IGF-1	12.55	11.50	0.712
ERα	13.86	10.29	0.211
EGFR	9.64	13.36	0.193
HER-2	12.27	10.73	0.606

Table shows mean ranks and significance value (*p*-value) of each gene expression based on Mann-Whitney test.

Table V. *Significance of differences in gene expression between tamoxifen resistant and tamoxifen sensitive ERα-positive breast cancer.*

Gene expression	Tamoxifen-resistant (Tam-R1 and TamR2) group (n=23)	Tamoxifen-sensitive group (n=69)	*p*-Value
IGF-1R	44.98	47.01	0.752
IGF-1	31.59	51.47	0.002
ERα	35.74	50.09	0.026
EGFR	48.25	42.56	0.361
HER-2	40.75	47.67	0.282

Table shows mean ranks and significance value (*p*-value) of each gene expression based on Mann-Whitney test.

were also lower in the Tam-R2 group compared to the tamoxifen sensitive group (p=0.006) but in the Tam-R1 group, this difference did not reach statistical significance (p=0.057). When compared to the tamoxifen sensitive group, ERα mRNA levels were lower only in the Tam-R2 group (p=0.033) (Table VII) but not in the Tam-R1 group (p=0.224) (Table VI).

EGFR mRNA levels were significantly higher in the Tam-R2 group compared to the tamoxifen sensitive group (p=0.045) (Table VII). When the Tam-R1 group was compared to the tamoxifen sensitive group, there was no difference in *EGFR* expression (p=0.535) (Table VI). When the tamoxifen resistant groups (whether analysing Tam-R1 and Tam-R2 cases together or separately as Tam-R1 or Tam-R2 subgroups) were compared to the tamoxifen sensitive group, there was no difference in *IGF-1R*, EGFR or *HER-2* mRNA levels (see Table V, VI and VII).

Correlation patterns between IGF-1, IGF-1R, ERα EGFR and HER-2 gene expressions in tamoxifen resistant and tamoxifen sensitive breast cancer tissue. The IGF-1 mRNA levels weakly correlated with those of *IGF-1R* in the tamoxifen resistant group (r=+0.424, p=0.020). There was no correlation between *IGF-1* and *IGF-1R* mRNA levels in the tamoxifen sensitive group (p=0.147). *IGF-1R* did not have any significant correlation with ERα, *EGFR* or *HER-2*

Table VI. *Significance of differences in gene expression between Tam-R1 and tamoxifen sensitive ERα-positive breast cancers.*

Gene expression	Tam-R1 group (n=11)	Tamoxifen-sensitive group (n=69)	*p*-value
IGF-1R	43.23	40.07	0.675
IGF-1	28.15	42.47	0.057
ERα	32.59	41.76	0.224
EGFR	34.68	39.15	0.535
HER-2	35.23	41.34	0.415

Table shows mean ranks and significance value (*p*-value) of each gene expression based on Mann-Whitney test.

Table VII. *Significance of differences in gene expression between Tam-R2 and tamoxifen sensitive ERα-positive breast cancers.*

Gene expression	Tam-R2 group only (n=12)	Tamoxifen sensitive group (n=69)	*p*-Value
IGF-1R	35.58	41.94	0.387
IGF-1	23.75	44.00	0.006
ERα	27.63	43.33	0.033
EGFR	50.82	36.42	0.045
HER-2	35.27	41.33	0.420

Table shows mean ranks and significance value (*p*-value) of each gene expression based on Mann-Whitney test.

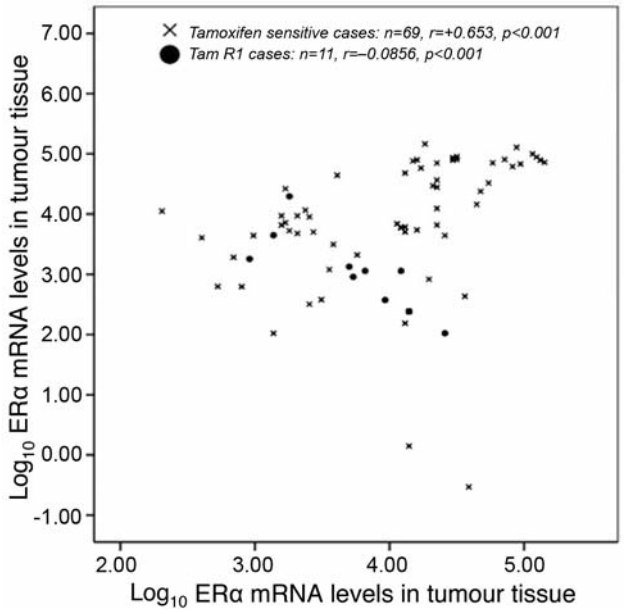

Figure 5. *Relationship between ERα and IGF-1 mRNA expression levels in Tam-R1 and tamoxifen sensitive tumours.*

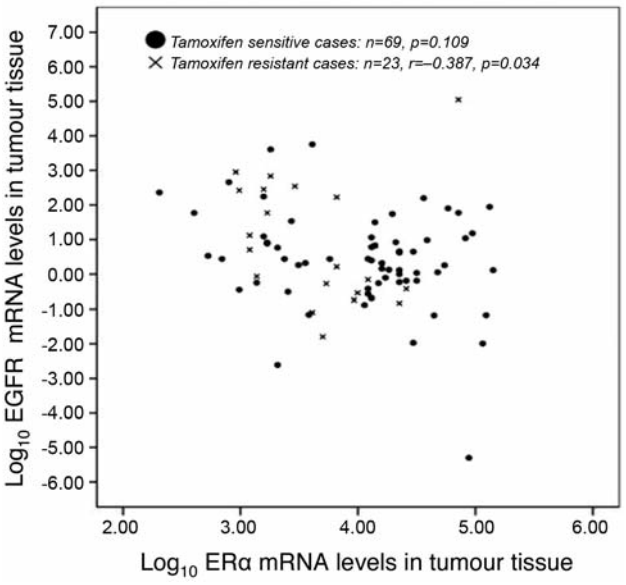

Figure 6. *Relationship between EGFR and ERα mRNA expression levels in tamoxifen resistant and tamoxifen sensitive tumours.*

in tamoxifen resistant groups (p=0.334, 0.065 and 0.461 respectively). *IGF-1* strongly correlated with ERα in the tamoxifen sensitive group (r=+0.653, p<0.001 but this correlation was not present in the tamoxifen-resistant groups (p=0.254). A further subanalysis of Tam-R1 cases only revealed that there was in fact a strong inverse correlation between *IGF-1* and *ERα* in the tamoxifen resistant tumours (r=–0.856, p<0.001) (Figure 5). We found that there was a weak inverse correlation between *ERα* and *EGFR* mRNA levels in the tamoxifen resistant group (r=–0.387, p=0.034) but not in the tamoxifen sensitive group (p=0.109) (Figure 6). *HER-2* did not correlate with any other gene expression in either groups.

Discussion

Reports on whether *IGF-1R* is elevated or decreased in anti-oestrogen-resistant breast cancer cell lines are conflicting. Nicholson *et al*. showed that endocrine–responsive cell lines expressed considerable levels of total and activated *IGF-1R* and that treatment with tamoxifen led to a significant drop in *IGF-1R* levels (20). In the tamoxifen resistant cell line, the *IGF-1R* levels and activation substantially recovered (20). This suggests that *IGF-1R* expression and activity are maintained in tamoxifen resistant phenotype. This was consistent with Pariosot *et al*. who found that *IGF-1R* expression in tamoxifen resistant cell lines was up-regulated

in response to oestrogen (23). However, Brockdorff *et al*. found that in six different anti-oestrogen-resistant breast cancer cell lines, there was a loss in *IGF-1R* levels compared to their parent cell line. Also noteworthy was that the restoration of *IGF-1R* levels was associated with regained sensitivity to anti-oestrogen treatment (3). In another study, anti-oestrogen resistant ZR-75-1 breast cancer cell line showed a decrease in *IGF-1R* expression compared to its anti-oestrogen-sensitive parental cell line (30). We aimed to determine if *IGF-1R* expression was up- or down-regulated in our tamoxifen resistant series.

In addition to *IGF-1R*, the local tissue *IGF-1* ligand expression should also, in theory, lead to stimulation of breast cancer cells *via IGF-1R* activation. Parisot *et al*. showed that tamoxifen resistant cell lines responded more to *IGF-1* stimulation than did the parental cell line (23). As yet, no other studies have specifically looked at the role of *IGF-1* expression in breast cancer tissue in tamoxifen resistance. We aimed to investigate whether there is a difference in *IGF-1* expression between tamoxifen sensitive and resistant breast cancer tissue.

ERα expression has long been considered an important factor in determining the response of *ERα*-positive breast carcinomas to endocrine/anti-oestrogen therapy (25). Down-regulation of *ERα* expression can contribute to *de novo* anti-oestrogen resistance (6, 12). The loss of *ERα* has been reported in a few breast cancer cell line studies; ZR-75 derived 9a1 cell lines become *ERα*-negative in the presence of tamoxifen but revert back to *ERα*-positive when tamoxifen is removed and oestrogen is reintroduced (31). In fulvestrant resistant MCF-7-r, T47D-r and ZR-75-r breast cancer cell lines, *ERα* protein is completely lacking and only minimal amounts of *ERα* mRNA are detectable (29). Another study showed that a fulvestrant resistant MCF-7 variant cell line showed a 90% decrease in both *ERα* protein and mRNA and this was accompanied by significant changes in the oestrogen-induced expression of *ERα* target genes (7). On the other hand, some studies have also shown the tamoxifen resistance phenotype in ERα-positive/HER-2 positive tumours to be unrelated to ERα levels (5). Despite that, many of the *in vitro* studies mentioned earlier suggested that *ERα* expression is one of the main determinants of the effectiveness to anti-oestrogen treatment. We would like to test this finding by measuring *ERα* expression in our series of *ERα*-positive breast cancer tissue in order to determine if it could be used to predict failure to tamoxifen treatment. We also aimed to see how *ERα* mRNA expression compares to *ERα* measured by immunohistochemistry (IHC) and Allred's scoring (1) in predicting time-to failure of tamoxifen treatment.

Several studies have established that overexpression of *EGFR* and *HER-2* in ERα-positive breast cancer cells can confer resistance to anti-oestrogens (16, 19). High levels of *HER-2* signalling have been associated with reduced tamoxifen efficacy (26). In addition, many studies have shown that the interaction between *ERα, EGFR* and *HER-2* receptor pathways can influence the sensitivity of breast cancer cells to anti-oestrogen treatment (5). A study on 222 breast cancer samples revealed that the expressions of *EGFR, HER-2* and *HER-3* were inversely correlated with ERα levels and overexpression of *EGFR, HER-2* and *HER-3* were associated with shorter overall survival (32). Dowsett *et al*. found that *ERα*-positive *HER-2* positive breast cancers have a lower expression of *ERα* compared to *ERα*-positive *HER-2*-negative breast carcinomas (5). In fact, HER-2 has been found to be inversely related to *ERα* in other studies involving breast cancer tissue specimens (2, 14). Because *ERα* expression is strongly related to response to hormone/anti-oestrogen therapy in primary and advanced breast cancer, reduced ERα or PR expression may be one mechanisms to explain the relative resistance of *HER-2/neu*-positive: HR-positive tumours to hormone therapy (14). We aimed to determine if *EGFR* and *HER-2* expression could be used to predict tamoxifen resistance and explore its correlation with ERα expression.

To date, most studies have shown that the EGFR pathway is the predominant growth factor receptor pathway in tamoxifen resistance compared to the *IGF-1R* pathway. Jones *et al*. showed the effects of treating tamoxifen resistant MCF-7 cell lines with various combinations of AG1024 (*IGF-1R* inhibitor), gefitinib (*EGFR* inhibitor), *IGF-1* and *EGFR* ligands. When the tamoxifen resistant cell line growth was treated with both AG1024 and then EGF ligands, the inhibitory effect of AG0124 was readily overridden by the addition of EGF ligands; however, the actions of gefitinib were only partially reversed by the addition of *IGF-1*. This suggests that *IGF-1R* signalling plays only a supportive role in the *EGFR/HER-2* axis in the growth stimulation of tamoxifen resistant breast cancer cells. However, a subsequent study by the same team on tamoxifen resistant and gefitinib-resistant MCF-7 cell line showed an increase in levels of *IGF-1R* and intracellular signalling molecules Akt and protein kinase C (which represent increase in growth factor receptor activity) (13). This shows that the *IGF-1R* can supersede the *EGFR* pathway in maintaining tamoxifen resistance when the EGFR pathway is deactivated. A study and review by Van Den Berg *et al*. stated that in tamoxifen sensitive ZR-75-1 cell lines, both *EGFR* and *IGF-1R* are co expressed but in the tamoxifen resistant variant, both *EGFR* and *IGF-1R* are inversely correlated (30). We aimed to look at the relationship between *IGF-1R* and *EGFR* in our tamoxifen resistant breast cancers compared to the tamoxifen sensitive cases.

We used clinical history to determine the sensitivity of *ERα*-positive breast cancer to tamoxifen. We looked at the gene expression pattern in *ERα*-positive breast cancer cells that would suggest *de novo* or acquired Tamoxifen-resistance.

For this purpose, we analysed our Tam-R1 and Tam-R2 groups together and separately to detect any difference in features between Tam-R1 and Tam-R2. In order to look at the difference in gene expression between tamoxifen resistant and tamoxifen sensitive tumours, we used a large number of ERα-positive tumours from patients who underwent surgery and adjuvant tamoxifen but did not develop recurrence or metastasis as tamoxifen sensitive cases. We are aware that it is not possible to confirm that all cases in this group are truly tamoxifen sensitive as some patients may be clear of residual disease after primary treatment. However, because the tamoxifen sensitive group consists of a larger number of cases compared to Tam-R1 and Tam-R2 group and 26 out of 69 cases in the tamoxifen sensitive group were LN-positive cases (high risk of developing distant metastasis without hormonal/tamoxifen treatment), we decided to use it as a 'control' group to compare with Tam-R1 and Tam-R2 tumours. An ideal study to investigate molecular pathways involved in tamoxifen resistance would be to recruit breast cancer patients being treated with primary tamoxifen treatment and obtain paired samples before starting tamoxifen and after development of tamoxifen resistance. The difficulty in obtaining ethics approval for such a study has been mentioned in other studies (9).

In theory, the *IGF-1* ligand should stimulate breast cancer cells *via* the *IGF-1R*, which may lead to anti-apoptosis and resistance to tamoxifen treatment. However, this study showed that an increase in tumour *IGF-1* mRNA level was associated with a longer period to tamoxifen failure. This suggests that higher *IGF-1* mRNA levels are associated with a lower tendency for breast cancer cells to develop tamoxifen resistance, which is contradictory to our hypothesis. However, in the tamoxifen sensitive group, there was a strong correlation between *IGF-1* and *ERα* but this correlation was not present in our tamoxifen resistant samples. In fact, further subanalysis of the Tam-R1 cases only showed that *IGF-1* inversely correlated with ERα mRNA levels which would suggest that the relationship between *IGF-1* and *ERα* expression changes on development of tamoxifen resistance.

In *ERα*-positive breast cancer, *IGF-1* is known to be a mediator of oestrogen stimulation and ERα tend to correlate with *IGF-1R* and *IGF-1* expression (8, 15). Our tamoxifen sensitive breast cancer patients had not been treated with tamoxifen and were still receiving oestrogen stimulation at the time of surgery for the breast cancer sample and this could explain the higher *IGF-1* mRNA levels in this group. As tamoxifen resistant breast cancer tumours become independent of oestrogen stimulation, it may be possible that this mechanism may be lost and this may explain why *IGF-1* levels were lower in the tamoxifen resistant group.

We did not find any association between *IGF-1R* mRNA levels and time-to-failure of tamoxifen treatment. We found that there was no difference in *IGF-1R* mRNA levels between tamoxifen resistant and tamoxifen sensitive groups. Other studies have shown conflicting results in which some report an overexpression of *IGF-1R* (20, 23) or an under-expression (30) of *IGF-1R* in tamoxifen resistant breast cancer cell lines. Hence, our results have not yielded any further progress on the subject. However, the role of activated *IGF-1R*, such as phospho-*IGF-1R* or its downstream signalling molecules such as IRS-1 and Akt/phosphor-AKT could be more representative of *IGF-1R* activity and expression (17, 18). More studies on *IGF-1R* and tamoxifen resistant breast cancers are needed.

The positive correlation between *IGF-1* and *IGF-1R* mRNA levels in the tamoxifen resistant samples suggests that both ligand and receptor are co-expressed or may even exist in a positive feedback loop. This would imply that at least at the gene transcription level, the lower *IGF-1* mRNA in tamoxifen resistant samples could in turn lead to lower *IGF-1R* mRNA levels. More research is required to confirm the status of *IGF-1* and *IGF-1R* in tamoxifen resistant breast cancer.

One of the important results of this study is that a higher *ERα* mRNA level was associated with longer time-to-failure of tamoxifen treatment. Based on Allred's scoring, we aimed to see if *ERα* protein expression could be used to determine time-to-failure of tamoxifen treatment. Unlike *ERα* mRNA, ERα protein measured using IHC and Allred scoring did not predict time-to-failure of tamoxifen treatment. In addition, the tamoxifen resistant (Tam-R1 and Tam-R2) groups had significantly lower *ERα* mRNA levels compared to the tamoxifen sensitive group. This finding is consistent with numerous studies which have found that *ERα* expression was a possible determinant of effectiveness to anti-oestrogen treatment (6, 7, 12, 25, 29). These findings also highlight the need to use other techniques for measuring *ERα* status as compared to the IHC method commonly employed in current clinical practice. As mentioned in the introduction, unpredictability of anti-oestrogen responses may lie in the current *ERα* assays used, which measures only an initial component of the oestrogen responsive pathway. Paik *et al*. measured a profile of genes using RT-PCR and used a recurrence algorithm to predict the outcome of patients with tamoxifen treated node-negative breast cancers. They found that the *ERα* protein expression data did not have the same predictive information as the gene expression profile scoring (22).

Even though *EGFR* expression was not associated with time-to-failure of tamoxifen treatment, we found that patients who developed recurrence or metastasis (the Tam-R2 group) had higher *EGFR* mRNA levels compared to patients who did not (*i.e*. the tamoxifen-sensitive group). This has been a consistent feature in many studies which show that tamoxifen resistant breast carcinomas often overexpress *EGFR* (2, 4, 14, 20). One study even pointed out that *EGFR* expression could be used to predict tamoxifen resistance (2).

Another significant finding from our study is that *EGFR* was inversely correlated with *ERα* mRNA levels in the tamoxifen resistant group. This finding would mean that as *EGFR* expression increases in tamoxifen resistant samples, there should a corresponding decrease in *ERα* expression. This relationship would be consistent with those found by other studies (2, 4, 14, 20). As mentioned earlier, we found that the *ERα* mRNA level could be a possible predictor of anti-oestrogen responsiveness of breast carcinomas. The inverse correlation between *EGFR* and *ERα* mRNA was not present in the tamoxifen sensitive group, which would mean that this mechanism may be an important step in developing a tamoxifen resistant phenotype. We did not find any significant results for *HER-2* mRNA levels in our series.

Conclusion

We found that both *IGF-1* and *ERα* expressions are raised in breast cancer cases which were likely to develop tamoxifen resistance. Measuring *ERα* mRNA using RT-PCR may be more sensitive in determining tamoxifen sensitivity than measuring *ERα* using IHC. *IGF-1R* expression is not altered in tamoxifen resistant tumours. *EGFR* is overexpressed in breast cancers that are likely to become tamoxifen resistant. The inverse correlation between *EGFR* and *ERα* expression could be a feature of tamoxifen resistant tumours. This supports other reports that *EGFR* expression may be responsible for tamoxifen resistance and this may be mediated by decreasing *ERα* expression.

Competing Interest

The authors declare that they have no competing interests.

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