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Epidermal growth factor receptor/HER2/insulin-like growth factor receptor signalling and oestrogen receptor activity in clinical breast cancer

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Abstract

Breast cancer models of acquired tamoxifen resistance, oestrogen receptor $(ER)^+/ER^-$ *de novo* resistance and gene transfer studies cumulatively demonstrate the increased importance of growth factor receptor signalling, notably the epidermal growth factor receptor (EGFR)/HER2, in tamoxifen resistance. Our recent *in vitro* studies also suggest that EGFR signalling productively cross-talks with insulin-like growth factor receptor (IGF-1R) and, where present, activates ER on key AF-1 serine residues to facilitate acquired tamoxifen-resistant growth. This paper presents our immunohistochemical evidence that EGFR/HER2 signalling (i.e. transforming growth factor (TGF)α, EGFR and HER2 expression; phosphorylation of EGFR, HER2 and ERK1/2 MAP kinase) is also prominent in clinical *de novo* resistant and modestly increased in acquired tamoxifen-resistant states, suggesting that anti-EGFR/HER2 strategies may prove valuable treatments. Primary breast cancer samples employed were obtained for (1) patients subsequently treated with tamoxifen for advanced disease where endocrine response and survival data were available and (2) ER^+ elderly patients during tamoxifen response and relapse. We also present our clinical immunohistochemical findings that IGF-1R expression, its phosphorylation on tyrosine 1316, and also phosphorylation on serine 118 of ER are not only prominent in ER^+ tamoxifen-responsive disease, but are also detectable in ER^+ *de novo* and acquired tamoxifen-resistant breast cancer, where there is evidence of EGFR/ER cross-talk. Our data suggest that agents to deplete effectively ER or IGF-1R signalling may be of value in treating ER^+ *de novo*/acquired tamoxifen resistance in addition to tamoxifen-responsive disease *in vivo*. IGF-1R inhibitors may also prove valuable in ER^- patients, since considerable IGF-1R signalling activity was apparent within ~50% of such tumours.

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Introduction

The most widely used and best-studied antihormonal approach in clinical breast cancer is the antioestrogen tamoxifen (Johnston *et al*. 2003). Tamoxifen competitively inhibits binding of oestrogen to its target oestrogen receptor (ER) and brings about a receptor conformational change blocking AF-2 function. This inhibits proliferation of ER^+ breast cancer cells, resulting in therapeutic responses in ~60% of ER^+ patients. Tamoxifen has proved invaluable in the management of ER^+ advanced disease, prolongs survival in the adjuvant setting, and is of preventative value in women at high risk of developing the disease. However, a

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pervading problem that limits the effectiveness of this agent and ultimately confers poorer prognosis is resistance. Resistance is apparent in a proportion of patients *de novo*, and is acquired by many responders during tamoxifen treatment. While lack of the target receptor underlies *de novo* tamoxifen resistance in 30% of breast cancer, ~40% of ER^+ patients also fail to respond *de novo*. Many of these ER^+, resistant patients are refractory to additional endocrine manipulation *de novo* (endocrine insensitive); however, others respond to such treatment. Acquired tamoxifen resistance is commonly associated with retention of functional ER, such that subsequent endocrine response after tamoxifen relapse can occur.

It is important that we decipher the regulatory pathways underlying the various forms of tamoxifen resistance if we are to treat effectively or even prevent this adverse state. Of some interest is ER and also growth factor receptor signalling pathways, notably the epidermal growth factor receptor (EGFR)/HER2 and insulin-like growth factor receptor (IGF-1R) networks. Interestingly, emerging experimental data suggest that such pathways are interactive (Nicholson *et al*. 2004*a*). The importance of this signalling is being assessed by various methods applied to human breast cancer *in vitro* and xenograft models of *de novo* and acquired tamoxifen resistance. Judicious use of pharmacological agents in these various models is helping determine the relevance of any identified elements to resistant growth and to establish their future potential as therapeutic targets. However, this strategy must be underpinned by equivalence in the target signalling pathway elements between experimental systems and clinical breast cancer material. This paper describes how our immunohistochemical profiling of key EGFR/HER2 signalling elements in acquired, tamoxifen-resistant, clinical breast cancer and in *de novo* tamoxifen-resistant versus responsive samples from Nottingham City Hospital has obtained data complementary to tamoxifen-resistant models. Importantly, we also assess whether there is clinical evidence of a role for activation of IGF-1R and, where retained, of ER in resistance.

Experimental models reveal increased importance of EGFR/HER2 signalling in acquired tamoxifen resistance versus the tamoxifen-responsive phase, with profiling of expression/activity of this pathway also suggesting relevance to clinical disease

In order to detail the underlying molecular biology of acquired resistance to endocrine agents, our group has previously established several *in vitro* breast cancer models, including acquired tamoxifen-resistant cells (TAMR) developed from the parental ER^+ breast cancer cell line MCF-7. TAMR cells emerge after ~3 months' culture of MCF-7 in the presence of 4-hydroxytamoxifen, despite an initial endocrine-responsive (i.e. growth-suppressive) phase, and such cells remain highly ER^+. Our study of acquired TAMR cells has demonstrated expression of key EGFR ligands, some elevated, as well as increases in the EGFR and HER2 receptors versus tamoxifen-responsive MCF-7 cells (Knowlden *et al*. 2003, Nicholson *et al*. 2004*a*). There also appears to be increased signalling activity through these receptors in TAMR cells, as evidenced by enhanced EGFR/HER2 heterodimerisation and receptor phosphorylation, as well as increased activation of the downstream kinases ERK1/2 MAP kinase (MAPK) and AKT, elements previously implicated in tumour proliferation and cell survival. In total, our data have conclusively demonstrated that autocrine growth factor signalling through EGFR/HER2 is dominant in promoting acquired tamoxifen-resistant growth. Of course, such signalling may not be relevant across all acquired resistant models. However, our data are certainly complemented by a battery of previous *in vitro* gene transfer studies suggesting a causative association between tamoxifen resistance and increased EGFR/HER2/MAPK/AKT signalling, and also by the phenotypes of several additional acquired tamoxifen-resistance models (Benz *et al*. 1993, Kurokawa & Arteaga 2003). The EGFR-selective tyrosine kinase inhibitor gefitinib and also the humanised HER2-directed antibody herceptin can subvert such signalling and decrease growth of our acquired TAMR cells *in vitro*, confirming the key importance of EGFR/HER2 signalling (Knowlden *et al*. 2003, Nicholson *et al*. 2004*a*). Inhibitors targeting downstream elements within this signalling pathway, notably inhibitors of MAP kinase kinase 1 (MEK1), are also valuable. This is in contrast to the lack of significant impact of such agents in tamoxifen-responsive MCF-7 cells, where EGFR/HER2 signalling and MAPK activation are minimal and EGFR/HER2 signalling does not comprise a dominant growth mechanism.

In order to determine whether there is clinical evidence supportive of the importance of EGFR/HER2 signalling in acquired tamoxifen resistance, we have immunohistochemically examined a series of samples from ER^+ patients treated in the Nottingham Breast Unit who underwent sequential needle-core biopsies of their breast tumours before treatment, during primary tamoxifen treatment (20 mg/day) and subsequently at the time of disease progression (Gee *et al*. 1999, Kenny

et al. 2001). The series provided an invaluable opportunity to compare the tumour phenotype during the tamoxifen-responsive and acquired-resistance phases *in vivo*. The patients were elderly (>70 years), at stages I–II or locally advanced, where primary hormone therapy was deemed an appropriate initial treatment. Study of sequential biopsies was conducted in full accordance with the ethical principles for clinical trials, and every patient had given informed consent to undergo serial tumour biopsies as part of larger studies evaluating the phenotypic changes in breast carcinomas on primary endocrine therapy (Kenny *et al*. 2000, 2001). Response was assessed at 6 months by UICC criteria (CR or PR (complete or partial response); SD or PD (static or progressive disease)), and all patients remained on tamoxifen until progression of disease. We were successful in demonstrating modest, but nevertheless significant, increases in several key components of EGFR/HER2 signalling after acquisition of resistance by some patients (Gee *et al*. 1999, Nicholson *et al*. 2004*a*). Common phenotypic features of tamoxifen relapse samples included elevated expression of transforming growth factor-alpha (TGF-α) and its target receptor EGFR. Our study also observed some increases in cytoplasmic HER2 in relapse samples versus response. The EGFR signalling increases observed were relatively modest when compared with the obvious changes in our acquired tamoxifen-resistant model. Indeed, we found that their detection required highly sensitive immunohistochemistry, and they were observed only when relapse samples were compared with samples taken during tamoxifen response rather than before treatment. Such requirements may explain why some groups have been unable to detect EGFR increases in equivalent clinical material where conventional analysis of growth factor receptor expression positivity is employed. In support of our data, however, HER2 amplification has been reported by others on relapse with antihormonal treatment, while one study showed that ~25% of patients treated with first-line hormonal therapy converted from serum HER2 negative to positive on relapse (Lonn *et al*. 1996, Lipton *et al*. 2003). Verification of the role of modestly increased EGFR signalling in acquired tamoxifen resistance should be achievable from the various ongoing clinical trials employing gefitinib in such breast cancers (Nicholson *et al*. 2004*b*). In this regard, Robertson *et al*. (2003) have shown that gefitinib responses can occur despite this modest EGFR signalling in acquired tamoxifen-resistant, clinical material: clearly, overexpression of EGFR signalling elements is not essential for recruitment of this pathway to tamoxifen-resistant growth.

Our clinical profiling of EGFR signalling to date has failed to address whether the receptors are heterodimerised or activated in resistance, an approach that would provide cellular evidence that the receptors are pathologically or therapeutically relevant. Preliminary immunoprecipitation studies in our laboratory have now demonstrated that EGFR/HER2 heterodimerisation is detectable in fresh frozen clinical breast cancer material. Moreover, it is now possible immunohistochemically to apply antibodies directed to phosphorylated 'activated' forms of EGFR and HER2 and their downstream signalling elements to archival, formalin-fixed, paraffin-embedded, clinical breast cancer material. While technically demanding and requiring rigorous quality assessment, this approach is beginning to yield rewarding data that appear to complement our model systems of acquired resistance. Using such assays, we have detected activity of EGFR and HER2 in the tumour cell plasma membranes and cytoplasm of the majority of the clinical, acquired tamoxifen-resistant samples examined to date (Fig. 1a and b). The activity of HER2 appears to exceed that of EGFR, although the levels versus the responsive phase of the disease have not as yet been addressed. We have also immunohistochemically examined MAPK activation in this sequential clinical sample series, employing an antibody to the dually phosphorylated pTEpY region within the catalytic core of active MAPK, and have shown significantly increased MAPK phosphorylation in ~80% of ER^+ patients on acquisition of tamoxifen resistance versus tamoxifen response (Hutcheson *et al*. 2003*a*) (Fig. 1c). Increased activity of the additional MAPK family members Jun kinase and p38 was also apparent in ~30% of these samples, in agreement with other studies (Johnston *et al*. 1999). Cumulatively, these emerging data profiling ligands, receptors and the activity of downstream pathway components suggest that EGFR/HER2 signal transduction is of some importance to the acquired resistant state *in vivo* as well as *in vitro*.

Experimental models reveal EGFR/HER2 signalling can also contribute to ER^+ *de novo* tamoxifen resistance and ER^+ endocrine insensitivity, and even promote ER negativity, with profiling of expression/activity of this pathway also suggesting relevance to clinical disease

There are few models of ER^+ tamoxifen *de novo* resistance, although those that are available invariably indicate relevance of markedly elevated EGFR/HER2 signalling.

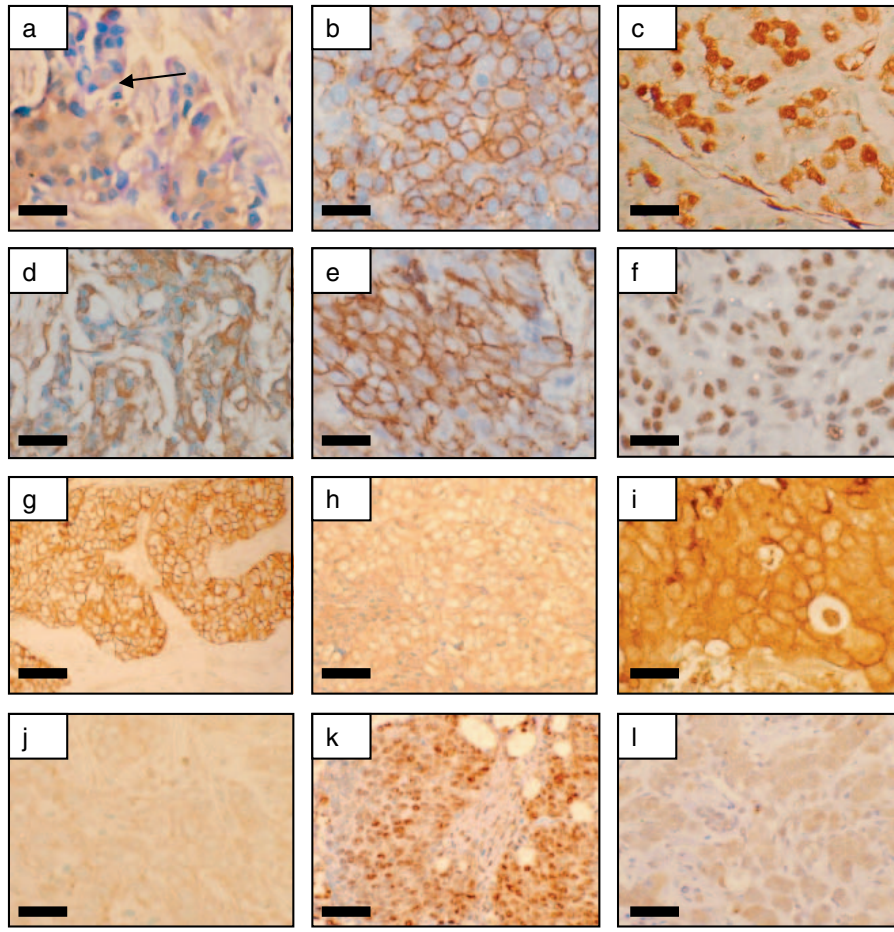

Figure 1 Immunohistochemical staining for (a) phosphorylated EGFR (arrow marks plasma membrane staining), (b) phosphorylated HER2, (c) phosphorylated ERK1/2 MAP kinase, (d) IGF-1R expression, (e) phosphorylated IGF-1R and (f) phosphorylated serine 118 ER in acquired tamoxifen-resistant, clinical breast cancer. Substantial immunohistochemical staining in primary breast cancer from patients who received endocrine therapy for advanced disease for (g) IGF-1R expression or (i) phosphorylated IGF-1R versus poor stainers (h and j respectively), as well as an ER^+ phosphorylated serine 118 ER-rich tumour (k) versus absence of staining in ER^- disease (l). Scale bars in a–f, i and j = 20 μm; g, h, k and l = 40 μm.

MCF-7 cells stably transfected with HER2 (MCF-7/HER2-18) are *de novo* resistant to tamoxifen, in contrast to their low-expressing, responsive MCF-7 counterparts (Benz *et al.* 1993, Shou *et al.* 2004). ER^+ BT474 cells endogenously expressing high levels of HER2 are also *de novo* tamoxifen resistant (Kurokawa & Arteaga 2003), while our recent studies have shown only poor tamoxifen responses in ER^+ T47D cells that have obvious expression of both EGFR and HER2. Growth of these various cell lines is commonly inhibited by EGFR, HER2 or downstream kinase blockade, suggesting an important growth contribution for this pathway in ER^+, *de novo* tamoxifen-resistant models (Kurokawa & Arteaga 2003, Shou *et al.* 2004). While the *de novo* tamoxifen-resistant BT-474 and T47D cells retain substantial ER expression, tamoxifen-resistant, HER2-transfected MCF-7/HER2-18 cells exhibit reduced levels of ER versus their parental MCF-7 cells. This is in keeping with the reported clinical association between HER2 overexpression and reduced ER level, and the poorer response and survival of ER^+ patients exhibiting lower steroid hormone receptor levels (Konecny *et al.* 2003). Importantly, however, EGFR/HER2 signalling remains dependent on the ER in MCF-7/HER2-18 cells, as evidenced by their retained sensitivity to oestrogen deprivation (Shou *et al.* 2004). Productive interplay between these mitogenic pathways thus appears to be important in promoting resistance to tamoxifen (in addition to the impact of any ER reduction).

However, our group has demonstrated that complete dislocation from ER is also possible when growth factor signalling is substantially elevated. Exposure of MCF-7 cells to the ERBB ligand heregulin, which markedly upregulates MAPK and AKT signalling, is associated with lack of response to all endocrine manipulation (Nicholson *et al*. 2004*c*). Similarly, second-line response to faslodex in our acquired TAMR cells can be abrogated by further hyperactivation of EGFR/HER2 signalling by several exogenous EGFR ligands (Hutcheson *et al*. 2003, Nicholson *et al*. 2004*c*). Thus, extreme growth factor signalling not only promotes experimental *de novo* tamoxifen resistance in ER^+ cells, but also, under certain (as yet poorly defined) conditions, may be able to signal in an ER-independent manner and promote ER^+ endocrine insensitivity. Such increased growth factor signalling may even reduce ER expression/function to promote ER^- growth experimentally. Certainly, the ER^- phenotype *in vitro* commonly exhibits marked EGFR overexpression coupled with hyperactivation of downstream MAPK, and in some ER^- cell lines the EGFR signalling pathway is growth contributory (Biswas *et al*. 2000). Several groups have shown that exposure of ER^+ cells to various growth factors, including EGF, IGFs and heregulins, can, under poorly defined circumstances, promote signalling that is able to decrease ER expression (Stoica *et al*. 2000). Moreover, promotion of ER negativity appears achievable *in vitro* when hormone-responsive cells are subject to sustained EGFR signalling during very prolonged ER blockade. This is exemplified by our MCF-7 model that, after extended culture with the pure antioestrogen faslodex, gains EGFR signalling but completely lacks ER, a feature irreversible on antioestrogen withdrawal (Nicholson *et al*. 2004*c*). Further supportive evidence can be drawn from transfection studies where constitutively active components of EGFR/HER2 signalling introduced into ER^+ MCF-7 cells (notably MEK1 or ligand-stimulated EGFR) have all been shown to deplete ER and impair its function (Oh *et al*. 2001). In this regard, we have shown that constitutive upregulation of MEK1 in MCF-7 cells substantially hyperactivates MAPK and in parallel markedly decreases ER and promotes loss of the oestrogen-regulated gene progesterone receptor (PgR) (Nicholson *et al*. 2004*c*).

There is substantial evidence to indicate that these experimental observations may be paralleled in clinical disease. Our group has consistently shown that tumours with exaggerated EGFR/HER2 signalling elements on presentation are commonly *de novo* tamoxifen resistant with a poor prognosis (Lovekin *et al*. 1991, Nicholson *et al*. 1993, 1994*a*, *b*, 2004*a*, Gee *et al*. 2001). An historical series from Nottingham City Hospital formed the focus for these studies, comprising frozen as well as formalin-fixed, paraffin-embedded, primary breast cancers from patients who received endocrine therapy (90% tamoxifen) for locally advanced primary or metastatic disease (Gee *et al*. 2001). Patients had no previous adjuvant endocrine/cytotoxic therapy. Quality of response at 6 months, time to disease progression and survival from initiation of therapy, as well as extensive tumour marker data, were available. Our previous immunohistochemical studies showed that tamoxifen-responsive primary breast cancers generally expressed low EGFR/HER2 levels (Nicholson *et al*. 1993, 1994*a*). TGFα was also barely detectable in the CR/PR cohort (Nicholson *et al*. 1994*b*). Monitoring of MAPK phosphorylation revealed only low levels in tamoxifen-responsive tumours, also equating with improved patient survival (Gee *et al*. 2001). These clinical data appear to mirror observations made in MCF-7 cells, where EGFR/HER2 signalling is minimal and does not comprise a dominant regulatory pathway. In contrast, there was significantly increased expression of EGFR and TGF-α in ER^+, *de novo* tamoxifen-resistant patients, with a weaker association for HER2 (Nicholson *et al*. 1993, 1994*a*, *b*). Increased MAPK activity was also noted in ~80% of these tumours, with multivariate analysis revealing significant associations between increased MAPK activity, earlier relapse on therapy, and poorer survival in ER^+ disease (Gee *et al*. 2001). While other groups have suggested that HER2-overexpressing patients are less likely to respond to tamoxifen (Houston *et al*. 1999), an association with resistance in ER^+ disease has remained controversial (Elledge *et al*. 1998). However, Houston *et al*. (1999) and, more recently, Arpino *et al*. (2004) showed that HER2 and EGFR equate with tamoxifen resistance in ER^+ tumours, while Dowsett *et al*. (2001) demonstrated an impeded antiproliferative response to endocrine strategies in $ER^+/HER2^+$ disease. As in model studies where we have hyperactivated signalling pathways using exogenous growth factors, some ER^+, EGFR/HER2-overexpressing patients are likely to be completely endocrine insensitive; nevertheless, studies from Ellis *et al*. (2003) demonstrate that a proportion of these patients retain sensitivity to oestrogen deprivation, as noted in the MCF-7/HER2-18 model.

Particularly striking was our observation that 95% of ER^- clinical breast cancers exhibited marked EGFR plasma membrane immunostaining with a prominent inverse association apparent between EGFR overexpression and ER (Nicholson *et al*. 1994*a*, Sharma *et al*.

1994). HER2 overexpression occurred in ~40% of ER^- patients. Overexpression of these receptors was associated with aggressive disease features and poorer patient prognosis (Lovekin *et al*. 1991, Nicholson *et al*. 1993). Extensive data from many groups confirm that increased EGFR and HER2 are prominent in the ER^- phenotype (Tsutsui *et al*. 2002, Konecny *et al*. 2003), invariably being associated with poorer outlook. While EGFR activity has not yet been examined, our recent application of a phospho-specific HER2 antibody to the Nottingham primary breast cancer historical series has shown significant association with HER2 overexpression and high-grade, ER^- disease. Thor *et al*. (2000) have similarly used a further phospho-specific HER2 antibody to show relationships between increased activity, proliferation and poor prognosis in node-positive patients. We also noted that increased MAPK activation is extremely common in ER^-/$EGFR^+$ patients. Not surprisingly, therefore, elevated staining for MAPK activity was a significant feature of *de novo* resistance, shortened time to disease progression and poorer survival in clinical breast cancer (Gee *et al*. 2001). These findings in clinical ER^- tumours mirror experimental observations indicating a role for markedly elevated EGFR/HER2 signal transduction in such cells, and therefore raise the intriguing possibility that such signalling might even underlie ER loss in some ER^- patients.

Experimental models reveal IGF-1R signalling contributes to tamoxifen-responsive and acquired resistant growth, with a possible role in ER^+ and ER^- *de novo* resistance, while IGF-1R expression/activity may also be relevant to clinical disease

IGF-1R comprises a dominant signalling pathway promoting the growth and survival of tamoxifen-responsive breast cancer cells *in vitro*. MCF-7 cells exhibit prominent expression and activation of IGF-IR at their plasma membranes and are growth inhibited by specific IGF-1R inhibitors, including AG1024 (Nicholson *et al*. 2004*a*). In such cells, IGF-1R signalling is highly interactive with ER signalling. For example, oestrogens induce components of IGF-1R signalling, while IGFs prime kinase-mediated ER phosphorylation to enhance ER transcriptional activity (Hamelers & Steenbergh 2003). As a result, IGFs are powerfully mitogenic in tamoxifen-responsive cells and act to support oestrogen/ER-promoted growth. Because of this close cross-talk, tamoxifen diminishes IGF-1R signalling at multiple levels as part of its response mechanism (Guvakova & Surmacz 1997). However, recent data indicate that while IGF-1R expression remains somewhat lower than in MCF-7, IGF2 ligand and hence receptor activation is recovered in our acquired TAMR cells, and these cells retain growth sensitivity to AG1024 (Nicholson *et al*. 2004*a*). Interestingly, IGFs appear able to activate not only their target receptor, but also the EGFR, with both events blocked by IGF-1R inhibition (Nicholson *et al*. 2004*a*). While controversial, additional model systems tentatively implicate IGF-IR signalling in tamoxifen resistance. Growth of the tamoxifen-resistant MCF-7/5-23 cell line was significantly inhibited by the IGF-1R antibody αIR-3 (Parisot *et al*. 1999), while further acquired tamoxifen-resistant models have been described where proliferation is dependent on IGF signalling (Wiseman *et al*. 1993). There thus appears to be emerging evidence of a role for IGF-1R in acquired tamoxifen-resistant as well as -responsive cells. In contrast, there are only sparse and inconclusive data regarding the role of IGF-1R in experimental *de novo* resistance. With regard to ER^+, *de novo* resistant cells, our poorly responsive T47D line has detectable levels of IGF-1R expression and activity, as has the MCF-7/HER2-18 cell line, although the role of IGF-1R in growth, to our knowledge, remains unexplored. In ER- cells, IGF-1R levels are generally decreased (Bartucci *et al*. 2001), although the IGF2 ligand may be constitutively expressed. While a role for IGF-1R in ER^- metastasis is likely, its growth impact in such cells remains controversial.

It is notable that there are no conclusive clinical data examining IGF-IR expression in relation to tamoxifen response or resistance. Moreover, no studies have examined IGF-1R activation in this context, or profiled IGF-1R expression/activation at the time of acquisition of tamoxifen resistance. We have therefore developed new immunohistochemical assays for IGF-1R expression (IGF-1Rα antibody; Biotechnology Inc, Santa Cruz, CA, USA) and activity (polyclonal antibody detecting Y1316 IGF-1R phosphorylation). pY1316 phosphorylation contributes to IGF-1R-mediated mitogenesis/tumourigenesis, and, importantly, the pY1316 antibody does not cross-react with activated insulin receptor. In the first instance, IGF-1R expression and activation assays were applied to the Nottingham historical, paraffin-embedded, primary breast cancer series ($n=64$) to address relationships with tamoxifen response/*de novo* resistance (Gee *et al*. 2003). For these new studies (and also for examination of ER phosphorylation: see below) performed in the historical breast cancer series, approval for use without

the need for further patient consent was encompassed under an approved ethics application to the Nottingham Research Ethics Committee 2 (C1080301). Assays employed optimised antigen retrieval and sensitive, peroxidase-labelled polymer detection and were validated with preimmune serum or blocking peptide controls. Staining was principally at the plasma membrane for receptor expression and activation, and this was subsequently assessed by Hscore analysis (termed IGF-1Rm and pIGF-1Rm respectively). Immunostaining for these parameters was detected in 98% of patients. However, the signal was heterogeneous, and so tumours were classified as 'rich' or 'poor' for IGF-1Rm expression or pIGF-1Rm (defined by using the median HScores as cutoffs) (Fig. 1g–j). Previous RT-PCR from our group has also demonstrated ubiquitous IGF-1R expression, as well as extensive IGF-1R ligand expression. By the Mann – Whitney U test, IGF-1Rm immunohistochemical expression was significantly higher in ER^+ ($P=0.019$) (Fig. 2) or PgR^+ tumours than their receptor-negative counterparts, with a weak association between elevated IGF-1Rm and lower grade. While there was no correlation with HER2, IGF-1Rm expression was higher where EGFR was at lower levels. These data agree with reports that IGF-IR expression is enriched in ER^+, well-differentiated clinical breast cancer (Happerfield *et al*. 1997). In contrast, our monitoring of IGF-1R activity failed to reveal any association with steroid receptors, EGFR, HER2 or grade. Examination versus tamoxifen response revealed that tumours from responding patients were generally IGF-1Rm rich (with no difference in expression across CR, PR or SD), while those with progressive disease were frequently IGF-1Rm poor. Kaplan–Meier univariate survival analyses (log rank test) revealed that IGF-1Rm-rich patients had a significantly increased time to progression (TTP) versus IGF-1Rm-poor patients ($P=0.003$) (Fig. 3a). This was maintained as a strong trend in the ER^+ cohort. Parallel analysis of IGF-1R activity revealed no relationship with quality of response, but a weak trend for pIGF-1Rm-rich patients to have an increased TTP ($P=0.099$) (Fig. 3b) that was maintained in ER^+ disease. While not apparent for IGF-1R expression, there was also a trend for pIGF-1Rm-rich patients to exhibit increased survival from initiation of therapy. Cumulatively, these new immunohistochemical data for IGF-1R expression and activity suggest that increased IGF-1R signalling may be relevant to growth of tamoxifen-responsive disease in the clinic, mirroring observations in MCF-7 cells where IGF-1R is a dominant pathway that supports oestrogen/ER driven growth.

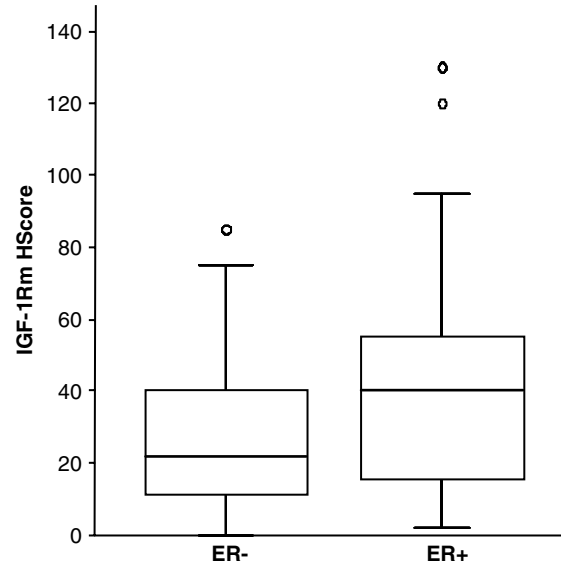

Figure 2 Box-plot illustrating IGF-1R plasma membrane (IGF-1Rm) immunohistochemistry assessed by HScore analysis in ER^- vs ER^+ clinical breast cancer (Mann – Whitney U test, $P=0.019$).

However, the relationship between IGF-1R activity and tamoxifen response in this patient series was imperfect. pIGF-1Rm was at detectable levels in all non-responders and was furthermore enriched in ~50%, comprising both ER^+, *de novo* resistant (including EGFR overexpressors) and ER^- patients. Furthermore, our recent application of such assays to a small number of the ER^+, acquired tamoxifen-resistant series samples noted that IGF-1R expression and activity is readily detectable (Fig. 1d and e). In total, our studies indicate that IGF-1R may be functional in clinical, acquired tamoxifen resistance in the presence of modestly increased EGFR signalling, and potentially also in ER^+ and ER^-, *de novo* tamoxifen resistance where EGFR can be markedly overexpressed. These data suggesting an importance for IGF-1R signalling in tamoxifen-resistant growth *in vivo* appear to complement observations in our acquired TAMR cells where IGF-1R activity aids EGFR signalling and growth (Nicholson *et al*. 2004*a*). Interestingly, while IGF-1R signalling has never been previously looked at in acquired endocrine resistance in the clinic, it has been reported that increased IGF-1R signalling occurs in acquired radioresistance (Turner *et al*. 1997). If our clinical results are considered alongside the limited model system data, the importance of IGF-1R signalling to ER^+ or ER^-, *de novo* tamoxifen-resistant growth remains uncertain. In support of such a role, however,

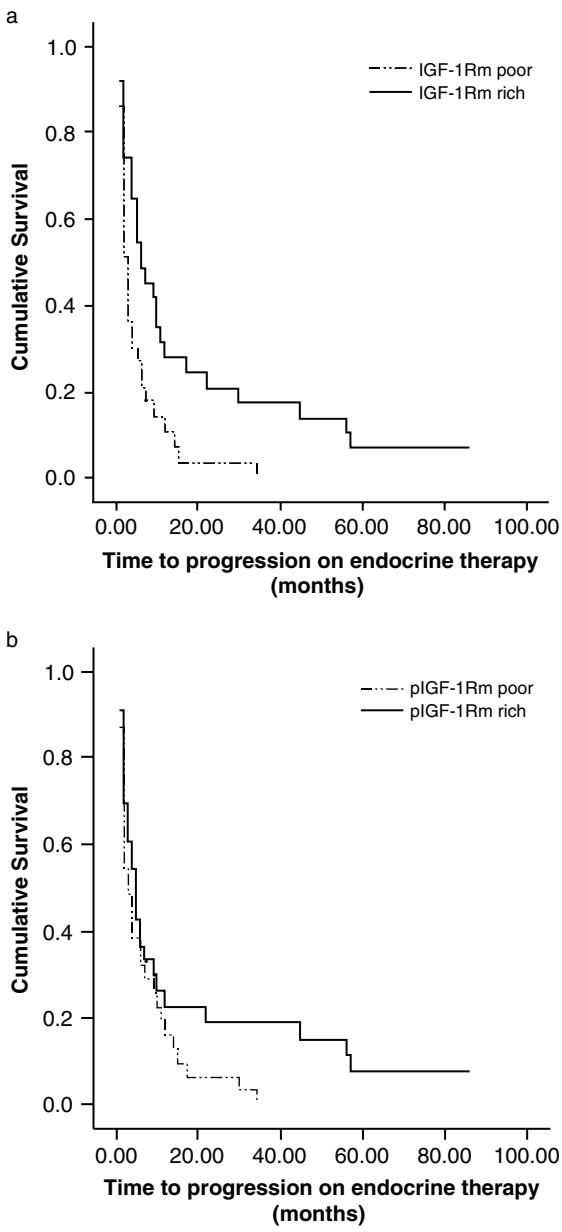

Figure 3 Kaplan–Meier curves (log rank test) for time to progression on endocrine therapy according to (a) IGF-1R plasma membrane expression (IGF-1Rm; $P=0.003$) or (b) phosphorylated IGF-1R plasma membrane staining status (pIGF-1Rm; $P=0.099$). 'Rich' or 'poor' status was categorised throughout this study by median HScores as cutoffs.

Railo *et al*. (1994) associated increased IGF-IR expression with shortened disease-free survival in ER^- patients, while higher levels of the IGF-IR signalling element insulin receptor substrate-1 predict increased recurrence in ER^+ disease (Rocha *et al*. 1997).

Experimental models reveal Ser118ER activation contributes to tamoxifen-responsive, acquired resistant and ER^+ *de novo* resistant growth, and may also be relevant to clinical disease

The human ER is a phosphoprotein hyperphosphorylated by oestrogens (Lannigan *et al*. 2003). Phosphorylation contributes to many aspects of receptor function, including subsequent coactivator recruitment and transcriptional activation of ER-regulated genes and, as a consequence, growth. While several sites on the receptor have been implicated according to cell context, serine 118 in the AF-1 region appears to be of some importance for ligand-dependent phosphorylation, perhaps mediated by the cyclin-dependent protein kinase Cdk7 (Chen *et al*. 2002). Indeed, in endocrine-responsive MCF-7 cells, oestradiol substantially activates Ser118ER.

However, several peptide growth factors and their intracellular signalling kinases, notably MAPK and AKT, are also able to phosphorylate ER on various AF-1 residues to promote ER transcriptional activity in a ligand-independent manner (Lannigan *et al*. 2003). This is interesting both in the context of our acquired TAMR cells and in the *de novo* tamoxifen-resistant MCF-7/HER2-18 line, both of which remain ER^+. While our acquired TAMR cells express equivalent levels of ER to the parental MCF-7 line, they exhibit increased phosphorylation on ER AF-1 residues, notably of serine 118 (pSer118ER). Activity of this site is regulated in TAMR cells by elevated EGFR/HER2-driven kinases, with gefitinib depleting its activity (Nicholson *et al*. 2004*a*). This contrasts with the lack of EGFR regulation of pSer118ER in MCF-7. EGFR/HER2-driven pSer118ER appears to facilitate the agonistic activity of the tamoxifen/ER complex in our acquired TAMR cells, promoting expression of ER-regulated EGFR ligands and IGF2 to complete the EGFR/HER2/IGF-1R growth-regulatory signalling loop (Hutcheson *et al*. 2003*b*, Nicholson *et al*. 2004*a*). In MCF-7/HER2-18, there is evidence that elevated HER2 signalling again contributes to tamoxifen agonism via a mechanism involving Ser118ER activity (and activity of the ER accessory protein AIB1) (Shou *et al*. 2004). Studies performed in various *de novo* tamoxifen-resistant, ER-transfected $HER2^+/EGFR^+$ ER^- lines further support the concept that increased EGFR/HER2 signalling can promote Ser118ER activity to subvert tamoxifen inhibitory effects (Kurokawa & Arteaga 2003), where such signalling is again abrogated in the various *de novo* resistant models by gefitinib. A key growth-promoting role for ER in the

context of these (and other) ER^+ acquired and *de novo* tamoxifen-resistant models is confirmed by their retained growth sensitivity to further endocrine challenge, cells being inhibited by faslodex treatment or oestrogen deprivation (Hutcheson *et al*. 2003*a*, Nicholson *et al*. 2004*a*,*c*; Shou *et al*. 2004). Thus, ER appears to be able to contribute to the growth of tamoxifen-responsive, acquired tamoxifen-resistant and *de novo* ER^+ resistant models.

Interestingly, in addition to responsive disease, there is evidence that ER remains important in some *de novo* tamoxifen-resistant and acquired resistant patients in the clinic. Expression of ER is prominent in some tamoxifen progressors *de novo*, while several studies indicate that the ER^+ status is generally retained on acquisition of tamoxifen resistance. In the Nottingham City Hospital sequential biopsy series, the tamoxifen-responsive phase was associated with decreased ER expression, but this was recovered at the time of tamoxifen relapse (Kenny *et al*. 2001). However, little is known about pSer118ER in breast cancer samples and its relationship to tamoxifen response/failure, and whether there is any supportive evidence that pSer118ER comprises a point of cross-talk with EGFR signalling in tamoxifen resistance *in vivo*. The immunohistochemical strategy we have undertaken to address these questions is as described for IGF-1R expression and activity with Nottingham clinical breast cancer, in this instance employing a sensitive assay for paraffin-embedded material with a monoclonal pSer118ER antibody (Cell Signalling Technology, Beverley, MA, USA; 16J4). While our initial data are presented here, we are currently expanding patient number and extending analysis of ER phosphorylation to encompass further AF-1 sites (e.g. serines 104/106 and 167). Staining with the pSer118ER antibody was principally nuclear; any cytoplasmic/plasma membrane staining failed to show meaningful clinical relationships. pSer118ER staining was heterogeneous between patients. Staining was directly associated with the ER^+ phenotype (Fig. 1k and l), and there was also a direct correlation between pSer118ER and PgR level, probably reflective of the requirement for ER activation to promote oestrogen-regulated gene expression. Occasional pSer118ER staining was observed in ER^- patients (also reported by Murphy *et al*. 2004*a*), although the significance of this observation remains uninvestigated. Tumours from responding patients generally had higher levels of pSer118ER than progressors, with no difference between CR, PR and SD. Kaplan–Meier univariate survival analyses revealed that pSer118ER-rich patients had significantly increased TTP. This association was maintained within

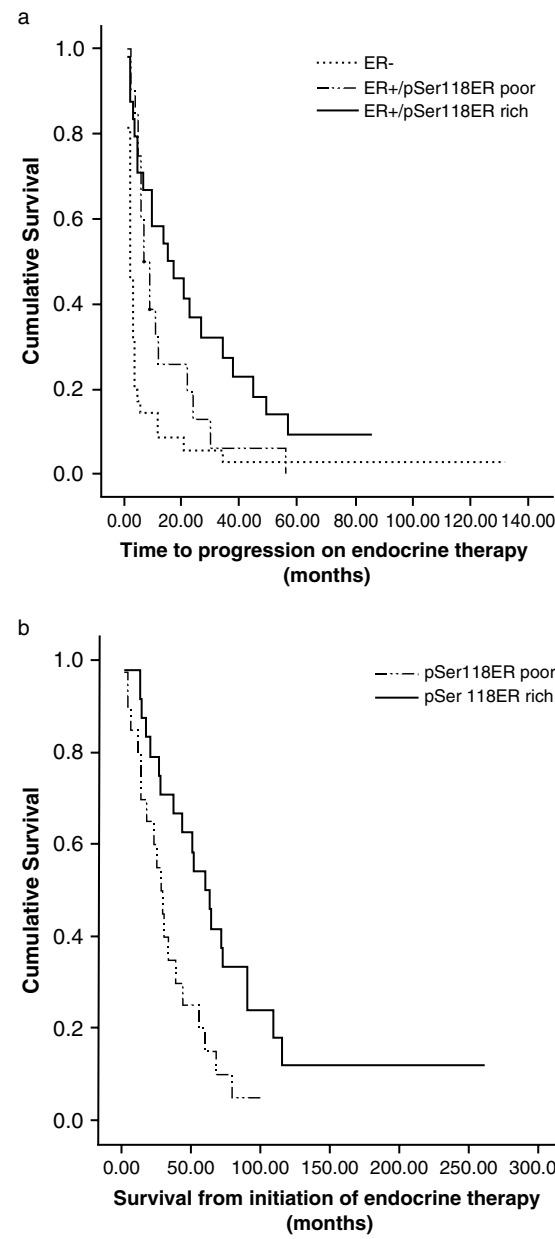

Figure 4 Kaplan–Meier curves (log rank test) for (a) time to progression on endocrine therapy comparing ER^- disease and ER^+ tumours classified as 'rich' or 'poor' stainers for phosphorylated serine 118ER (pSer118ER; $P=0.005$) and (b) survival from initiation of endocrine therapy in ER^+ patients stratified by pSer118ER status ($P=0.009$).

ER^+ patients, although ER^+/pSer118ER-poor patients still exhibited an improved TTP versus ER^- disease ($P=0.005$) (Fig. 4a). Interestingly, there were also a strong association between increased pSer118ER and improved survival from initiation of therapy that was again retained significantly in ER^+ disease ($P=0.009$)

(Fig. 4b). Prominent Ser118ER activity thus appears relevant to good prognosis and tamoxifen-responsive disease *in vivo*, and is reflective of functional, oestrogen-dependent ER signalling in such tumours, as in the MCF-7 model, where Ser118 is a dominant receptor site for hyperactivation by oestrogens during growth promotion. Our clinical data agree with Murphy *et al*. (2004*b*), who have shown that pSer118ER relates to longer disease-free survival, with a trend to overall survival, in ER^+, node-negative primary disease treated with adjuvant tamoxifen.

However, we again observed an imperfect relationship between Ser118ER activity and tamoxifen response. pSer118ER was at detectable levels in all ER^+ non-responders, with high levels in ~50%, including those overexpressing EGFR. Furthermore, pSer118ER was readily detectable in acquired tamoxifen-resistant samples (Fig. 1f). Interestingly, staining was also higher in $ER^+/EGFR^+$ than $ER^+/EGFR^-$ patients, with a direct association between EGFR and corresponding pSer118ER level. Murphy *et al*. (2004*a*) reported a direct correlation between pSer118ER and MAPK activity, and although this was not apparent in the Nottingham series, we did note a direct association between pSer118ER and AKT activity in ER^+, *de novo* resistant patients. These data parallel observations in our acquired TAMR model, where EGFR-driven kinase activation of Ser118ER comprises an integral part of the resistant growth mechanism, and also mirrors signalling reported in ER^+ MCF-7/HER2-18, *de novo* tamoxifen-resistant cells (Nicholson *et al*. 2004*a*, Shou *et al*. 2004).

Cumulatively, our data suggest that ER activity may remain contributory to acquired tamoxifen-resistant and also ER^+, *de novo* tamoxifen-resistant growth *in vivo*, with some evidence of its regulation by EGFR signalling. Retained ER signalling does appear growth relevant to some acquired tamoxifen-resistant tumours *in vivo*, since responses to subsequent endocrine therapies (notably faslodex and aromatase inhibitors) are frequent (Johnston 2004). There are also emerging data showing that ER remains growth contributory in some ER^+, *de novo* resistant tumours. Preliminary studies have revealed that while $HER2^+$ or $EGFR^+$ patients are relatively resistant to tamoxifen *de novo*, they retain sensitivity to aromatase inhibitors (Ellis *et al*. 2003).

Summary and therapeutic implications

While generally low in tamoxifen-responsive patients, EGFR/HER2 signalling can be modestly increased in acquired tamoxifen-resistant disease and more substantially in ER^+ *de novo* resistance *in vivo*. This mirrors the following: 1. our acquired TAMR model, where such signalling provides a dominant growth mechanism; 2. the phenotype of some ER^+, *de novo* resistant models; 3. exogenous growth factor-promoted endocrine resistance *in vitro*. Anti-EGFR strategies or herceptin may thus be valuable in acquired/ER^+, *de novo* tamoxifen-resistant patients, where *in vitro* challenge with these agents is encouraging in this regard. Indeed, herceptin can be valuable in $HER2^+$, tamoxifen-resistant patients, while our recent phase II study demonstrates that gefitinib can promote worthwhile clinical remissions in ER^+, acquired tamoxifen-resistant tumours modestly expressing EGFR (Robertson *et al*. 2003).

IGF-1R signalling and pSer118ER are prominent in tamoxifen-responsive patients, but are detectable (sometimes elevated) in ER^+ non-responders, with additional activity apparent in acquired tamoxifen-resistant tumours. These findings complement model system data (where available) for the various disease states. It thus appears that these signalling elements are unlikely to provide accurate markers for tamoxifen response/failure in clinical disease. The prevalence of ER activity across the various disease states may result from its multiple regulatory inputs; that is, oestrogens in tamoxifen-responsive cells and EGFR-driven kinases in acquired/ER^+ *de novo* resistance, where in all instances ER activity appears fundamental to growth. IGF-1R signalling may be an important growth input not only for tamoxifen-responsive tumours, where it enhances oestrogen/ER signalling, but also in resistant states, where it may facilitate mitogenic EGFR signalling. Therapeutically, our new data suggest agents to deplete more effectively oestrogen/ER signalling, and also anti-IGF-1R strategies may prove valuable both in tamoxifen-responsive disease and in ER^+, *de novo*/acquired tamoxifen resistance *in vivo*. Our experiences with Faslodex or IGF-1R inhibitors *in vitro* support this concept, as do the responses to faslodex or aromatase inhibitors exhibited by some acquired tamoxifen-resistant patients (Johnston 2004) and, for the latter agents, in a proportion of ER^+, *de novo* tamoxifen resistance (Ellis *et al*. 2003).

Finally, EGFR/HER2 components are markedly overexpressed in ER^- patients, mirroring the ER^- phenotype *in vitro* as well as the most extreme growth factor signalling/transfection models. ER^- disease thus seems an obvious target for anti-EGFR/HER2 agents. However, IGF-1R signalling is also present in these tumours *in vivo*. While as yet not supported by model data, our clinical findings do suggest that this pathway should also be considered as a target in ER^- tumours.

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