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PRECLINICAL STUDY

Evaluation of IGF1R and phosphorylated IGF1R as targets in HER2-positive breast cancer cell lines and tumours

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Abstract Insulin-like growth factor-1 receptor (IGF1R) signalling is implicated in resistance to trastuzumab. However, the benefit of co-targeting HER2 and IGF1R has not been extensively studied, and the relationship between activated IGF1R and clinical response to trastuzumab has not been reported. This study aimed to evaluate the combination of trastuzumab with IGF1R tyrosine kinase inhibitors (TKIs) in a panel of HER2-positive breast cancer cell lines, and to examine the relationship between IGF1R expression and activation and response to trastuzumab in HER2-positive breast cancer patients. The anti-proliferative effects of trastuzumab combined with IGF1R TKIs BMS-536924 or NVP-AEW541 were measured in nine HER2-positive cell lines. IGF1R and phosphorylated IGF1R/insulin receptor (pIGF1R/IR) were measured by immunohistochemistry in 160 tumour samples from trastuzumab-treated patients (ICORG 06-22). The HER2-positive cell lines displayed varying sensitivity to IGF1R TKIs alone (IC_{50}s: 0.7 to >10 μM). However, when combined with trastuzumab, a significantly enhanced effect was observed in five cell lines treated with BMS-536924, and three with NVP-AEW541. While IGF1R levels correlated with reduced response to NVP-AEW541 alone, neither IGF1R nor pIGF1R were predictive of response to BMS-536924 or NVP-AEW541 in combination with trastuzumab. Low HER2 levels correlated with response to BMS-536924 in combination with trastuzumab. Akt levels correlated with improved response to trastuzumab and NVP-AEW541 ($P = 0.039$). Cytoplasmic IGF1R staining was observed in all tumours, membrane IGF1R was detected in 13.8 %, and pIGF1R/IR was detected in 48.8 %. Although membrane IGF1R staining was associated with larger tumour size ($P = 0.041$), and lower tumour grade ($P = 0.024$), no association between IGF1R or pIGF1R/IR and patient survival was observed. In conclusion, while neither IGF1R expression nor activation was predictive of response to trastuzumab, these pre-clinical data provide evidence that co-targeting HER2 and IGF1R may be beneficial in some HER2-amplified breast cancers.

Keywords HER2 · Insulin-like growth factor-1 receptor · IGF1R · Trastuzumab · Resistance · Breast cancer

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Introduction

HER2 gene amplification or overexpression occur in approximately 25 % of human breast cancers [1, 2]. Trastuzumab (Herceptin, Genentech), a humanized monoclonal antibody targeting HER2 [3], has shown activity both as a single agent and in combination with chemotherapy [4, 5]. However, less than 35 % of HER2-positive patients respond to trastuzumab as a single agent, and most who initially respond develop resistance within 24 months [6].

Many potential mechanisms of resistance to trastuzumab therapy in HER2-positive breast cancer have been proposed including altered signalling via non-HER RTKs such as insulin-like growth factor-1 receptor (IGF1R) (for review, see [6, 7]). Although lapatinib (Tykerb, GlaxoSmithKline), a dual kinase inhibitor targeting both HER2 and EGFR, has shown clinical benefit in some trastuzumab-refractory patients, resistance to lapatinib also occurs [8], and can be mediated by mechanisms common to and independent of those mediating trastuzumab resistance [9].

Overexpression and activation of IGF1R and elevated IGF ligand levels have been observed in a number of human cancers [10–12], and aberrant signalling of the IGF system has also been implicated in resistance to trastuzumab in preclinical models. Transfection of IGF1R into trastuzumab-sensitive SKBR3 cells conferred almost complete resistance to trastuzumab [13]. In a model of acquired trastuzumab resistance, BT474/HerR cells had 3-fold higher levels of IGF1R compared to parental BT474 cells, and were more resistant to trastuzumab [14]. Reducing IGF1R signalling with recombinant IGF binding protein 3 (rhIGFBP3) restored sensitivity to trastuzumab in both of these models, and also had significant anti-tumour effects on MCF7/HER2-18 xenografts [13, 14]. Similar improvements in response to trastuzumab in cell line models have been observed using a dominant negative form of IGF1R, an anti-IGF1R antibody αIR3, and small interfering RNA (siRNA) targeting IGF1R [15–17]. Previous work from our laboratory and others' also showed that inhibition of IGF1R with the tyrosine kinase inhibitor (TKI) NVP-AEW541 enhanced response to trastuzumab in SKBR3 and BT474 cell lines, and restored sensitivity to trastuzumab in trastuzumab-resistant SKBR3/Tr cells [17, 18]. Co-targeting HER-2 and IGF1R in cell line models of de novo trastuzumab resistance has not been previously reported.

Current data on IGF1R expression or activity and response to trastuzumab in clinical samples are inconsistent. Two studies of 26 and 72 tumours from HER2-positive metastatic breast cancer patients reported no correlation between IGF1R expression and response to trastuzumab [19, 20]. However, in another study of 77 HER2-positive metastatic breast tumours, while IGF1R expression alone was not predictive of response to combination trastuzumab plus chemotherapy, the combination of high IGF1R and low S6 ribosomal protein phosphorylation (p-S6) correlated with worse outcome [21]. A significant association between IGF1R expression and reduced response to combination trastuzumab plus vinorelbine was also observed in a study of 46 early stage HER2-positive breast cancers [22]. In a more recent study of breast cancer subtypes, IGF1R expression correlated positively with survival overall, but in the HER2 subgroup ($n = 226$), a trend towards unfavourable outcome was observed [23]. However, a recent study of 1,746 early stage HER2-positive breast cancer patients reported no difference in response to trastuzumab between IGF1R-negative and -positive patient groups [24]. To date, the relationship between activated or phosphorylated IGF1R (pIGF1R) and response to trastuzumab has not been examined. However, pIGF1R/insulin receptor (IR) levels (measured using an antibody that detects both phosphorylated IGF1R and IR) were higher in HER2-positive tumours than other breast cancer subtypes, and overall, pIGF1R/IR levels were associated with shorter survival [25], which is contrary to what has been reported for total IGF1R expression [23].

The aims of this study were to perform a systematic preclinical evaluation of trastuzumab combined with IGF1R TKIs in a panel of HER2-overexpressing breast cancer cell lines, and to examine the relationship between IGF1R expression and phosphorylation and response to trastuzumab in HER2-positive breast cancers.

Materials and methods

Cell lines and reagents

BT474, EFM-192A, HCC1419, HCC1569, HCC1954, MDA-MB-453, SKBR3, UACC-732 and UACC-812 were obtained and cultured as previously described [9]. IGF1R and pIGF1R levels were measured by ELISA and reported previously [9]. BMS-536924 (Bristol Myers Squibb) and NVP-AEW541 (Novartis) (10 mM) were prepared in dimethyl sulfoxide. Trastuzumab (Roche) (21 mg/ml) was purchased from St Vincent's University Hospital.

Proliferation assays

Proliferation was measured using an acid phosphatase assay. 3–5 × 10^3 cells/well were seeded in 96-well plates. Plates were incubated overnight at 37 °C, followed by addition of drug and incubation for 5 days. After washing with PBS, 10 mM paranitrophenol phosphate substrate (Sigma-Aldrich) in 0.1 M sodium acetate buffer with 0.1 % Triton X (Sigma-Aldrich) was added and incubated at 37 °C for 2 h. 50 µl of 1 M NaOH was added and the

absorbance was read at 405 nM (with reference wavelength 620 nM), as previously described [26].

Patient information

Formalin-fixed paraffin-embedded tumour samples were obtained from 160 HER2-positive breast cancer patients from St. Vincent's University Hospital, Dublin, St. James's Hospital, Dublin, and Cork University Hospital, Cork, Ireland (ICORG 06-22). Ethical approval was obtained from the ethics committee of each hospital. Clinicopathological features are detailed in Table 1. Data for progression-free survival were not available for these patients. Survival data was available for 94 patients. Of the 94 patients for whom data were available, 66 % (48/73) received concurrent chemotherapy plus trastuzumab (TCH, AC-TH or TH) and 34 % (25/73) received trastuzumab after chemotherapy (AC, CMF, FEC or TAC). All patient deaths recorded were breast cancer related.

Tissue microarray (TMA) construction and immunohistochemistry

TMAs were constructed using a Tissue Arrayer (Beecher Instruments); four cores (6 mm) were taken from each sample. Immunohistochemistry was performed using an Autostainer (DAKO Diagnostics). Deparaffinization and antigen retrieval were performed using Epitope Retrieval 3-in-1 Solution (pH 6) (DAKO) and the PT Link system (DAKO), whereby slides are heated to 97 °C for 40 min, then cooled to 65 °C. IGF1R staining was performed using rabbit anti-IGF1Rβ antibody (Cell Signalling Technology), and pIGF1R/IR staining was performed using rabbit anti-phospho-IGF1Rβ/IR, which detects phosphorylated forms of both IGF1R and IR (Cell Signalling Technology). Thyroid tissue and cell lines (MCF7, BT474) were used as positive controls for IGF1R and pIGF1R/IR staining. HER2 staining was performed using the HercepTest (DAKO), using positive controls included with the kit. A negative control of antibody diluent in place of the primary antibody was also tested. Slides were counterstained with haematoxylin (Sigma-Aldrich) for 5 min, rinsed with dH_2O, and dehydrated in alcohols (2 × 3 min each in 70 % IMS, 90 % IMS and 100 % IMS), cleared in xylene (2 × 5 min) and mounted using DPX mountant (Sigma-Aldrich). Expression levels were assessed by a pathologist (Dr. Susan Kennedy). Cytoplasmic IGF1R was scored based on staining intensity: negative (0), weak (1+), moderate (2+) and strong (3+). Membrane IGF1R was scored as positive or negative, and only recorded as positive when it clearly exceeded the cytoplasmic staining [27]. pIGF1R/IR membrane staining was scored as negative (0), weak (1+), moderate (2+) and strong (3+). HER2 levels were scored according to the HercepTest guidelines.

Table 1 Characteristics of HER2-selected breast cancer patients ($n = 160$)

	n	%
Menopausal status		
Pre	41	25.6
Peri	3	1.8
Post	67	41.9
Unknown	49	30.6
Tumour size		
≤2 cm	32	20.0
>2 cm	115	71.9
Unknown	13	8.1
Nodal status		
Neg	59	36.9
Pos	93	58.1
Unknown	8	5.0
Grade		
1	2	1.3
2	30	18.8
3	120	75.0
Unknown	8	5.0
Histology		
D	152	95.0
L	2	1.3
Other	3	1.9
Unknown	3	1.9
ER		
Neg	76	47.5
Pos	76	47.5
Unknown	8	5.0
PR		
Neg	65	40.6
Pos	30	18.8
Unknown	65	40.6

Statistical Analyses

Statistical analyses were performed using SPSS 18.0 (SPSS Inc). Spearman rank analyses were performed to evaluate associations between protein levels and response to inhibitors. Single factor analyses of variance (ANOVA) and Tukey's multiple comparison tests were performed to evaluate drug combinations compared to single treatments. Chi-squared tests and Student's *t* tests were used to evaluate associations between protein expression and patient clinicopathological parameters. Patient overall survival (OS) was considered the survival end point. The data were partitioned in three different ways based on the detection of

membrane IGF1R, cytoplasmic IGF1R and pIGF1R/IR. For membrane IGF1R and pIGF1R/IR, the data were separated into two groups based on positive and negative staining. For cytoplasmic IGF1R, the data were partitioned into two groups, the first with scores of 0, 1+ and 2+, and the second with scores of 3+. The survival curves were based on Kaplan–Meier estimates and the log-rank *P* value was calculated for difference in survival. The R package survival was used to calculate and plot the Kaplan–Meier survival curves (http://cran.r-project.org/).

Results

Sensitivity to IGF1R tyrosine kinase inhibitors in HER2-overexpressing breast cancer cell lines

Nine HER2-positive breast cancer cell lines with varying IGF1R levels were tested for sensitivity to two IGF1R TKIs (BMS-536924 and NVP-AEW541). The IC_{50} values for the TKIs range from 0.7 μM in the most sensitive cell line (UACC-812) to greater than 10 μM (Table 2). Response to the two TKIs differed in the cell lines, for example BT474 and HCC1419 showed sensitivity to NVP-AEW541 but resistance to BMS-536924 (IC_{50} values >10 μM). The HER2-positive cell lines display lower sensitivity to IGF1R inhibition than the HER2 non-amplified MCF7, which was used as a positive control, and showed significant sensitivity to both TKIs (BMS-536924 $IC_{50} = 0.16 \pm 0.02$ μM; NVP-AEW541 $IC_{50} = 0.14 \pm 0.03$ μM). Neither IGF1R nor pIGF1R levels (measured by ELISA) were predictive of response to BMS-536924 (IGF1R: $P = 0.175$; pIGF1R: $P = 0.912$) (Supplementary Table 1); however, high levels of IGF1R and pIGF1R were associated with higher IC_{50} values for NVP-AEW541, with p values approaching statistical significance (IGF1R: $P = 0.053$; pIGF1R: $P = 0.078$) (Supplementary Table 1). The relationship between response to the IGF1R TKIs and HER2, pHER2, PTEN, Akt and pAkt levels, and PI3K mutation status, which were previously measured in this panel of cell lines [9], were also examined. A positive association was observed between NVP-AEW541 IC_{50} values and Akt levels ($P = 0.053$, Supplementary Table 1). Trastuzumab-resistant cell lines (as defined by O'Brien et al. [9]) tended to show greater sensitivity to BMS-536924 ($P = 0.093$), but no significant relationship with sensitivity to NVP-AEW541 was observed ($P = 0.196$).

Table 2 IGF1R and pIGF1R levels as determined by ELISA in a panel of HER2-positive breast cancer cell lines, and IC_{50} values (±std dev) for IGF1R tyrosine kinase inhibitors BMS-536924 and NVP-AEW541

Cell line	IGF1R ng/ml	pIGF1R (relative)	BMS-536924 IC_{50} (μM)	NVP-AEW541 IC_{50} (μM)
UACC-732	9.0 ± 0.4	0.3 ± 0.1	5.1 ± 0.2	2.9 ± 0.8
UACC-812	8.0 ± 0.9	0.6 ± 0.1	1.9 ± 0.5	0.7 ± 0.1
HCC1569	6.4 ± 1.8	0.3 ± 0.1	1.2 ± 0.2	1.3 ± 0.2
BT474	3.7 ± 0.5	0.5 ± 0.1	>10[a]	2.6 ± 0.2
HCC1954	3.1 ± 0.4	0.5 ± 0.1	1.8 ± 0.2	2.2 ± 0.5
HCC1419	3.0 ± 0.4	1.0 ± 0.0	>10[a]	2.8 ± 0.5
MDA-MB-453	1.6 ± 0.2	0.1 ± 0.1	5.5 ± 0.5	3.5 ± 0.4
SKBR3	0.7 ± 0.1	0.3 ± 0.1	8.3 ± 1.0	4.2 ± 0.6
EFM-192A	0.6 ± 0.1	ND	10.2 ± 0.5	3.3 ± 0.8

ND not determined

[a] >10 indicates that an IC_{50} was not achieved using concentrations up to 10 μM

Co-targeting HER2 and IGF1R in HER2-overexpressing breast cancer cell lines

The HER2-overexpressing breast cancer cell lines were treated with increasing concentrations of trastuzumab, IGF1R TKI (BMS-536924 or NVP-AEW541) or a combination of trastuzumab with IGF1R TKI (Fig. 1). It was not possible to calculate combination index values for the combination treatments as the trastuzumab treatment does not produce an appropriate sigmoidal dose response curve. We classified the response to the combinations as enhanced or non-enhanced based on an additive or greater than additive effect (statistically significant) when compared to the single agents at a fixed dose (2.5 μM TKI and 2.5 μg/ml trastuzumab). Trastuzumab with BMS-536924 showed an enhanced response in five of the nine cell lines (UACC-732, UACC-812, HCC1569, MDA-MB-453 and EFM-192A) (Fig. 1, Supplementary Table 2). NVP-AEW541 with trastuzumab showed an enhanced response in three of the cell lines (UACC-732, MDA-MB-453 and EFM-192A) compared to either agent alone (Fig. 2, Supplementary Table 2). Neither IGF1R nor pIGF1R levels correlated with improved response to the combination treatments. Enhanced response to the combinations did not correlate with response to the single agent TKIs or to trastuzumab sensitivity in the panel of cell lines (Supplementary Table 1). High levels of HER2 correlated with poorer response to the trastuzumab combined with BMS-536924 ($P = 0.050$, Supplementary Table 1). High Akt levels correlated with improved response to NVP-AEW541 with trastuzumab ($P = 0.039$, Supplementary Table 1). PI3K mutation status, levels of PTEN, pAkt or pHER2 did not correlate with response to the combination treatments.

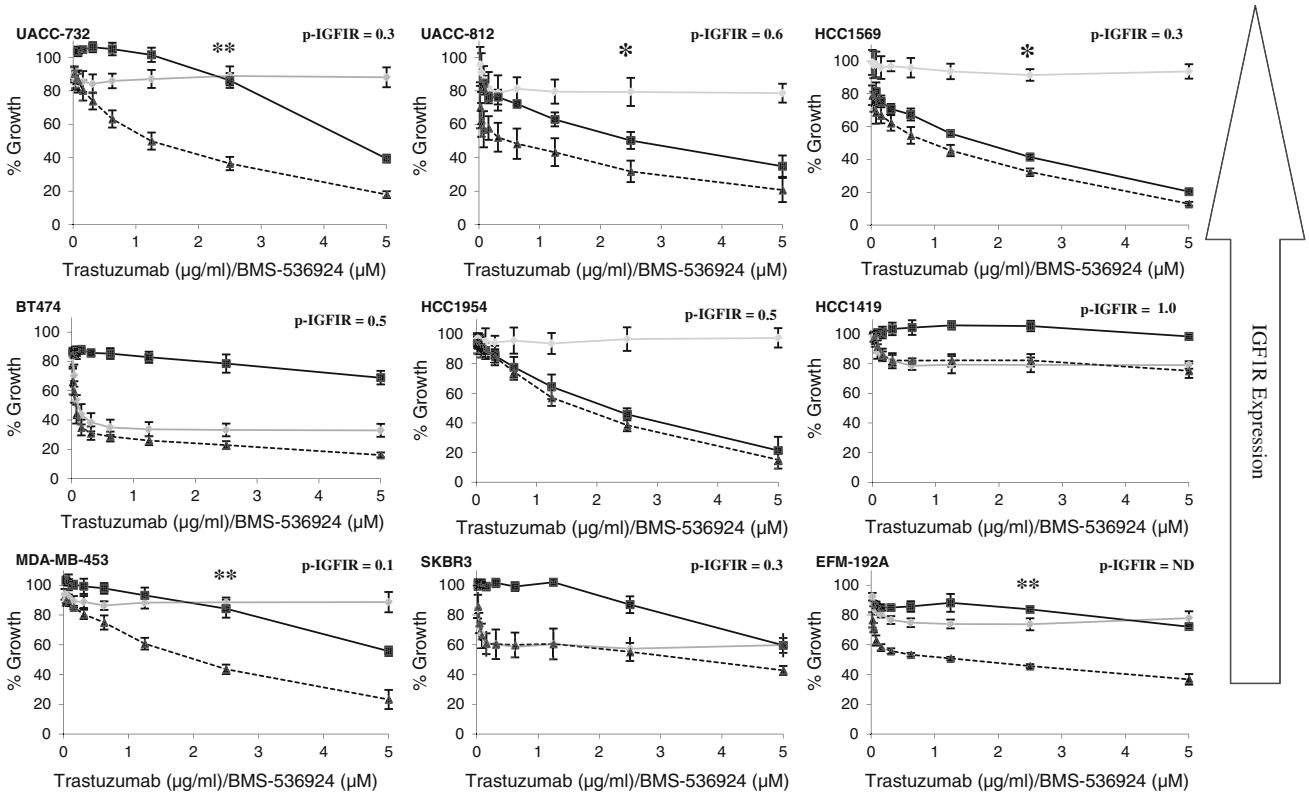

Fig. 1 Effect of trastuzumab (μg/ml) (*filled circle*), BMS-536924 (μM) (*filled square*), or a combination of trastuzumab and BMS-536924 (at a ratio of 1 μg/ml trastuzumab: 1 μM BMS-536294) (*filled triangle*) on proliferation of UACC-732, UACC-812, HCC1569, BT474, HCC1954, HCC1419, MDA-MB-453, SKBR3, EFM-192A cells. All assays were performed in 10 % FCS. *Error bars* represent the standard deviation of triplicate experiments. Relative phospho-IGF1R values, as measured by ELISA, are indicated

IGF1R and phosphorylated IGF1R/IR in HER2-positive breast tumours; association with clinicopathological parameters and response to trastuzumab

One hundred and sixty HER2-positive breast tumour samples were studied; 22 tumours (13.8 %) were scored 2+ for HER2 staining, and 138 (86.3 %) were scored 3+ (Table 3). While cytoplasmic IGF1R staining was observed in all 160 tumour samples studied, membrane IGF1R was detected in only 13.8 % of samples (22/160). Phosphorylated IGF1R/IR staining was detected in 48.8 % of samples (78/160) (Fig. 3; Table 3)

No relationship was observed between levels of membrane IGF1R, cytoplasmic IGF1R, phosphorylated IGF1R/IR or HER2 in these tumours (Supplementary Table 3).

Membrane IGF1R staining correlated significantly with larger tumour size (Chi-squared test, $P = 0.0414$) (Fig. 4a; Table 4), and with younger patient age at diagnosis (Student's 2-tailed t test, $P = 0.0479$) (Fig. 4b). Membrane IGF1R was also detected significantly more frequently in grade 2 tumours compared to grade 3 tumours (Chi-squared test, $P = 0.0239$) (Fig. 4c; Table 4). No association was observed between membrane IGF1R expression and patient menopausal status, nodal status, histological type, or ER or PR status (Table 4).

No significant associations were observed between cytoplasmic IGF1R or phosphorylated IGF1R/IR staining and any of the histopathological characteristics studied (Table 5, and Supplementary Tables 4 and 5). Phosphorylated IGF1R/IR was detected more frequently in node negative than node positive tumours, although this difference did not achieve statistical significance (Chi-squared test, $P = 0.0592$).

Survival data was available for 94 early stage breast cancer patients who received adjuvant trastuzumab-based therapy. Levels of membrane IGF1R, cytoplasmic IGF1R and phosphorylated IGF1R/IR did not correlate with patient survival in this cohort (mIGF1R: $P = 0.319$; cIGF1R: $P = 0.686$; pIGF1R/IR: $P = 0.504$) (Fig. 5).

Discussion

We examined inhibition of IGF1R in a panel of HER2-overexpressing breast cancer cell lines, and measured IGF1R expression and phosphorylation in HER2-positive breast cancer patients. We have previously reported that IGF1R does not correlate with resistance to trastuzumab in a panel of HER2-overexpressing cell lines [9]; however,

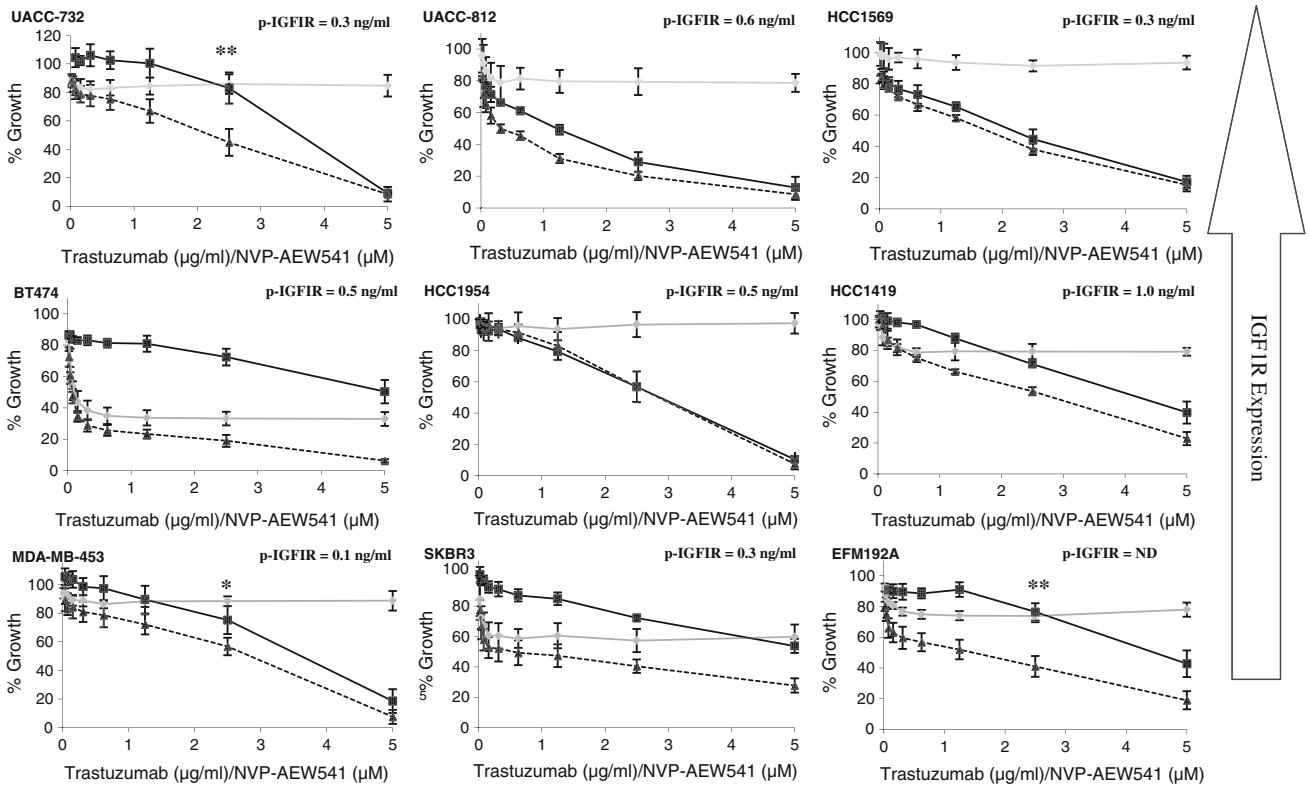

Fig. 2 Effect of trastuzumab (μg/ml) (*filled circle*), NVP-AEW541 (μM) (*filled square*), or a combination of trastuzumab and NVP-AEW541 (at a ratio of 1 μg/ml trastuzumab: 1 μM NVP-AEW541) (*filled triangle*) on proliferation of UACC-732, UACC-812, HCC1569, BT474, HCC1954, HCC1419, MDA-MB-453, SKBR3, EFM-192A cells. All assays were performed in 10 % FCS. *Error bars* represent the standard deviation of triplicate experiment. Relative phosphoIGF1R values, as measured by ELISA, are indicated

Table 3 Summary of membrane IGF1R, cytoplasmic IGF1R and phosphorylated IGF1R/IR staining in 160 tumours measured by IHC

Staining	*n*	%
Membrane IGF1R		
Negative	138	86.3
Positive	22	13.8
Cytoplasmic IGF1R		
1+	4	2.5
2+	64	40.0
3+	92	57.5
Phosphorylated IGF1R		
0	82	51.3
1+	34	21.3
2+	33	20.6
3+	11	6.9
HER2		
2+	22	13.8
3+	138	86.3

co-targeting HER2 and IGF1R was beneficial in three HER2-positive breast cancer cell lines, including two trastuzumab-resistant models [17]. We hereby extended these studies to examine IGF1R tyrosine kinase inhibition alone and in combination with trastuzumab in a panel of HER2-overexpressing cell lines.

Although BMS-536924 and NVP-AEW541 both target IGF1R and IR with similar potency (IC_{50}s 70–150 nM), response to the TKIs varied in the HER2-positive cell lines, with most of the cell lines displaying greater sensitivity to NVP-AEW541 than to BMS-536924. It is possible that inhibition of other targets may contribute to the differing sensitivity profiles. Based on in vitro kinase assays, NVP-AEW541 inhibits Tek, Flt1 and Flt3, and BMS-536924 inhibits FAK and Lck, at concentrations less than 1 μM. However, similar response profiles were observed when the two TKIs were combined with trastuzumab. Three cell lines showed enhanced response to both TKIs combined with trastuzumab (UACC-732, MDA-MB-453 and EFM-192A). Two additional cell lines (UACC-812 and HCC1569) showed improved response with both combinations but the difference was only statistically significant for the combination of trastuzumab with BMS-536924.

Although data for achievable concentrations in humans is not available for either of the TKIs, the IC_{50} values in the HER2-positive cell lines, albeit lower than in the positive control MCF7 cells, are within the range of concentrations

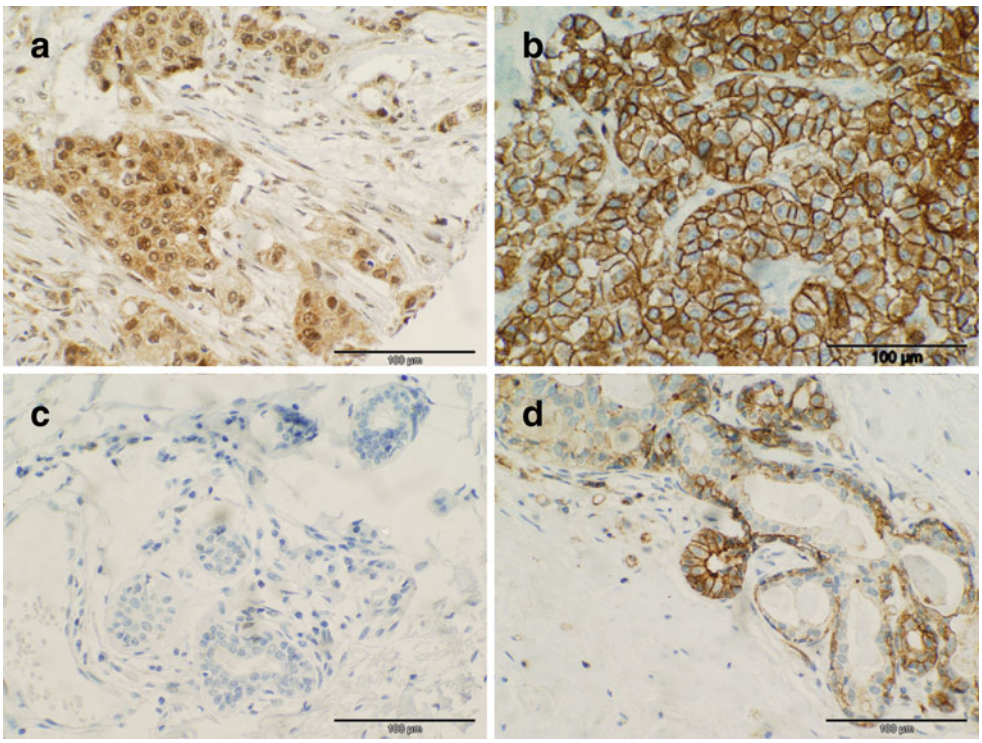

Fig. 3 Representative images of IGF1R and phosphorylated IGF1R/IR immunohistochemical staining: **a** IGF1R membrane negative, cytoplasmic 2+; **b** IGFIR membrane positive, cytoplasmic 1+; **c** phosphorylated IGF1R/IR negative, and **d** phosphorylated IGF1R/IR positive. All images were taken at 40× magnification

that have been reported for animal models (3–210 μM for NVP-AEW541 and 1-4 μM for BMS-536924) [28, 29].

The enhanced responses observed are consistent with previous reports combining HER2 and IGF1R antagonists in HER2-positive cells, where additive and synergistic effects on SKBR3 and BT474 cells, respectively, were observed [30, 31]. Though the combination effect was small in some of the cell lines in our study, three cell lines demonstrated an additive or greater than additive response to both combinations. Two of these cell lines are classified as trastuzumab- and lapatinib-resistant (UACC-732 and MDA-MB-453). This suggests a potential role for IGF1R targeting in tumours that are resistant to trastuzumab and lapatinib.

Modest associations were found between high IGF1R and pIGF1R levels and higher IC_{50}s for NVP-AEW541, suggesting that elevated IGF1R signalling may contribute to innate resistance to the inhibitor. No correlations between IGF1R or pIGF1R and response to BMS-536924 or to dual targeting with trastuzumab and the IGF1R TKIs were observed.

Alterations in the HER2 and PI3K/Akt signalling pathways have been implicated in resistance in HER2-overexpressing breast cancer (review see [6]). Therefore, we examined the HER2, phosphoHER2 and components of the PI3K/Akt pathway to determine if they play a role in response or resistance to the IGF1R TKIs or to dual targeting of HER2 and IGF1R. The cell lines which showed an enhanced response to trastuzumab plus BMS-536924 had lower levels of HER2 ($P = 0.05$), although no association with phosphoHER2 was observed. As was previously reported by O'Brien et al. [9], the levels of HER2 protein do not correlate with response to trastuzumab in the panel of HER2-amplified cells; thus, this correlation is unique to the combination of trastuzumab with BMS-536924 and suggests that a quantitative measurement of HER2 protein may be a predictive biomarker for trastuzumab combined with BMS-536924 in HER2-positive breast cancer.

We also found a correlation between Akt levels and higher NVP-AEW541 IC_{50} values, and a weak association with higher phosphoAkt in the panel of cell lines. Consistent with the associations with IGF1R and pIGF1R, this suggests that increased signalling through Akt may play a role in innate resistance to NVP-AEW541. Hägerstrand et al. [32] also reported that increased Akt signalling, mediated by PI3K mutation or ligand independent activation, were associated with resistance to NVP-AEW541 monotherapy in glioma cells. In contrast, we found that higher Akt correlated with improved response to combined trastuzumab and NVP-AEW541. Although the number of responsive cell lines is small (3), this may suggest that HER2-positive tumours with high levels of Akt may be more sensitive to NVP-AEW541 plus trastuzumab.

This study also aimed to investigate IGF1R expression and activity in HER2-positive breast tumour samples, and their relationship to clinical response to trastuzumab. We quantified membrane and cytoplasmic IGF1R staining separately.

Table 4 Relationship between membrane IGF1R staining and patient prognostic indicators

	n	Membrane IGF1R staining		
		No. positive	%	*P*
Menopausal status				
Pre	41	9	22.0	
Peri	3	0	0.0	0.0710
Post	67	5	7.5	
Tumour size (cm)				
≤2 cm	32	1	3.1	**0.0414**
>2 cm	115	20	17.4	
Nodal status				
Neg	59	11	18.6	0.2444
Pos	93	11	11.8	
Grade				
1	2	0	0.0	
2	30	9	30.0	**0.0239**
3	120	13	10.8	
Histology				
D	152	22	14.5	0.5611
L	2	0	0.0	
ER				
Neg	76	8	10.5	0.1666
Pos	76	14	18.4	
PR				
Neg	65	7	10.8	0.7166
Pos	30	4	13.3	

Chi-squared tests were performed using SPSS to determine *P* values

P values calculated by Chi-squared test are in bold

Cytoplasmic IGF1R was detected, at varying levels, in all 160 tumours, while membrane IGF1R was detected in 13.8 % of samples. Although IGF1R overexpression has been reported frequently in breast cancer, no universal scoring system has yet been implemented [12, 19, 21, 27, 33, 34]. Thus it is difficult to compare the frequency of IGF1R expression in our study with others'; however, membrane IGF1R expression was considerably lower in our study (13.8 %) than in non-selected breast cancer patient studies, where frequencies of between 44 and 70 % have been reported [27, 33–35]. This suggests that IGF1R membrane expression is downregulated in HER2-positive tumours. A recent study also showed decreased IGF1R in HER2-positive compared to other breast cancer subtypes [23].

Cytoplasmic localisation of IGF1R may be caused by: (i) increased cytoplasmic IGF1R following ligand binding and internalisation [36, 37], (ii) mutant receptors which fail to traffic to the membrane [38] and (iii) translocation to the nucleus [39]. Cytoplasmic expression of IGF1R in epithelial cells of normal breast tissue has been positively associated with subsequent risk of breast cancer [40]. However,

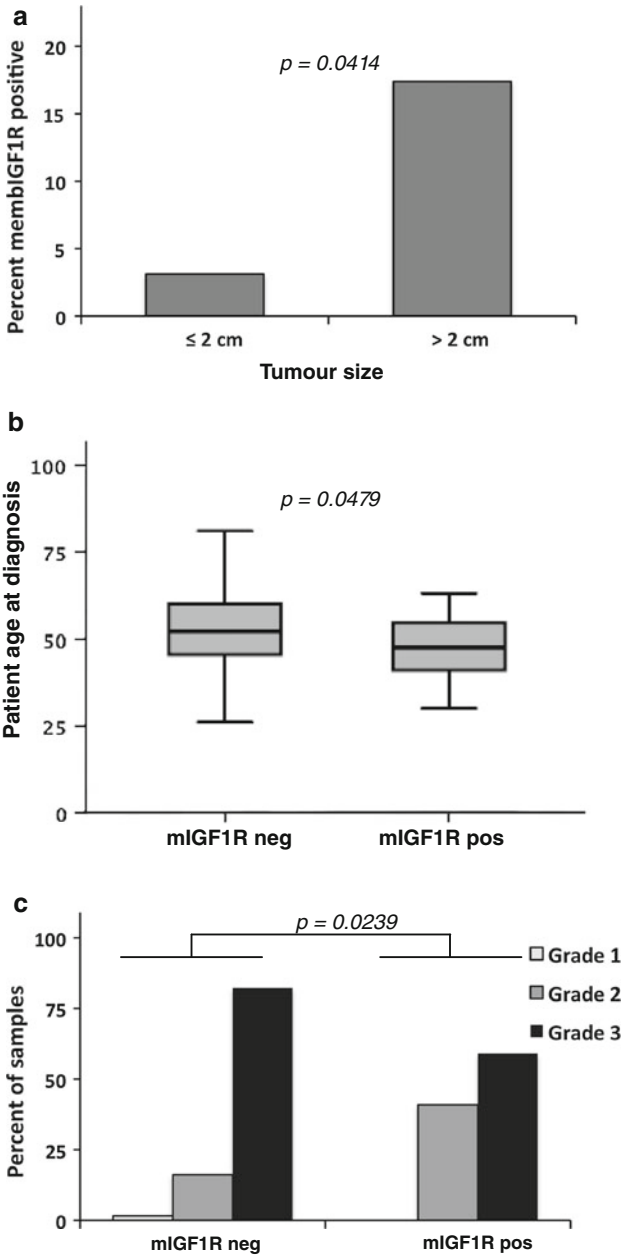

Fig. 4 Relationships between membrane IGF1R staining and patient characteristics: **a** Percent membrane IGF1R staining in tumours smaller ($n = 32$) and larger ($n = 115$) than 2 cm. The *P* value was determined using the Chi-squared test; **b** Boxplot showing the median patient age at diagnosis (line) of membrane IGF1R negative ($n = 20$) and positive ($n = 128$) tumours. The *boxes* represent upper and lower quartiles and the bars represent the range of values. The *P* value was determined using the Student's *t* test; **c** Percent of membrane IGF1R negative ($n = 22$) and positive ($n = 130$) tumours that are grade 1, 2 and 3. The *P* value was determined using the Chi-squared test

Hartog et al. [35] reported that cytoplasmic expression of IGF1R in ER-positive invasive ductal breast carcinomas is associated with a more favourable prognosis. In our study, cytoplasmic IGF1R staining did not correlate with pIGF1R/IR staining or with any prognostic indicators or outcome.

Table 5 Relationship between phosphorylated IGF1R/IR staining and patient prognostic indicators

	n	Phosphorylated IGF1R/IR staining		
		No. positive	%	*P*
Menopausal status				
Pre	41	20	48.8	
Peri	3	1	33.3	0.8648
Post	67	31	46.3	
Tumour size (cm)				
≤2 cm	32	16	50.0	0.5702
>2 cm	115	51	44.3	
Nodal status				
Neg	59	34	57.6	**0.0592**
Pos	93	39	41.9	
Grade				
1	2	1	50.0	
2	30	12	40.0	0.6655
3	120	59	49.2	
Histology				
D	152	75	49.3	0.1654
L	2	0	0.0	
ER				
Neg	76	39	51.3	0.4169
Pos	76	34	44.7	
PR				
Neg	65	31	47.7	0.4838
Pos	30	12	40.0	

Chi-squared tests were performed using SPSS to determine *P* values

P values calculated by Chi-squared test are in bold

Phosphorylated IGF1R levels were studied using an antibody that detects phosphorylated forms of both IGF1R and IR. pIGF1R/IR was detected in 48.8 % of tumours. pIGF1R/IR levels did not correlate with membrane or cytoplasmic IGF1R levels, which may be due to detection of phosphorylated forms of both IGF1R and IR. In a previous study of breast cancer subtypes, pIGF1R/IR levels were higher in the HER2-positive group than in luminal and triple negative breast cancer subtypes, and overall, pIGF1R/IR was associated with poor survival [25]. These and our data suggest that despite possible downregulation of IGF1R expression in HER2-positive cells, pIGF1R/IR signalling may be increased.

Membrane IGF1R correlated with larger tumour size and with younger patient age at diagnosis, suggesting that IGF1R may be associated with a negative prognosis. Membrane IGF1R expression has recently been associated with poor prognosis in ER-negative invasive breast cancers [35]. However, membrane IGF1R positivity also correlated with lower tumour grade in our study, which would be indicative of a favourable prognosis. Kaplan–Meier analysis of the 94 trastuzumab-treated patients for whom survival data was available revealed no correlation between membrane IGF1R, cytoplasmic IGF1R, or pIGF1R/IR and

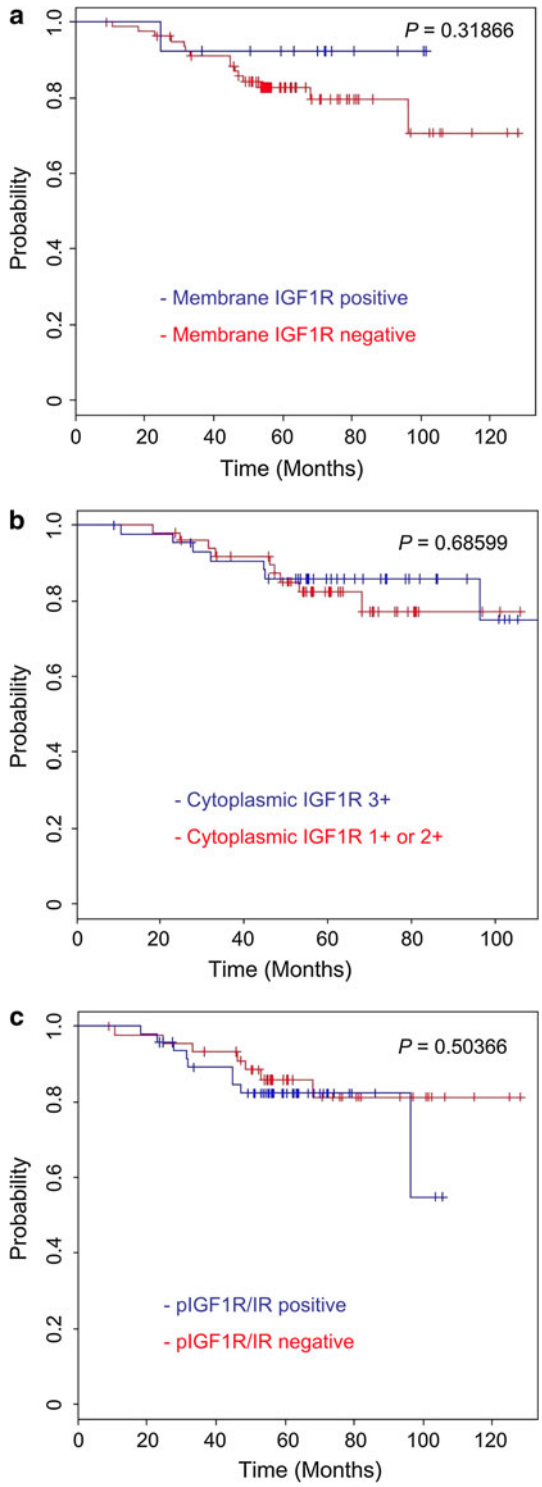

Fig. 5 Kaplan–Meier curves for **a** membrane IGF1R staining, **b** cytoplasmic IGF1R staining and **c** phosphorylated IGF1R/IR staining and overall survival in patients with early stage HER2-positive breast cancer treated with trastuzumab-based chemotherapy ($n = 94$)

patient survival. Other smaller studies have reported a similar lack of association between IGF1R expression and patient response to trastuzumab [19–21]. Furthermore, a recent study of 1,746 early stage HER2-positive breast cancer patients reported no difference in response to trastuzumab between IGF1R-negative and -positive patients [24]. In concordance with our data, the authors also found an association between IGF1R positivity and larger tumours, lower patient age, and lower % high grade. Collectively, these data provide convincing evidence that IGF1R is not an accurate predictor of prognosis, or resistance to trastuzumab, in HER2-positive breast cancer.

Although IGF1R levels in breast tumours did not predict response to adjuvant trastuzumab-based therapy, IGF1R status may change in the clinical course of breast cancer [41, 42]. Furthermore, treatment with trastuzumab may induce alterations in IGF1R signalling which may contribute to acquired trastuzumab resistance.

The in vitro data presented suggests that co-targeting HER2 and IGF1R may be beneficial in some HER2-positive tumours. Currently, phase I/II trials are underway combining IGF1R and HER2 therapies in metastatic HER2-positive breast cancer including: trastuzumab and BMS-754807, capecitabine and/or lapatinib and cixutumumab and trastuzumab and AMG479 (www.clinicaltrials.gov). The outcome of these studies should reveal the clinical benefits of co-targeting these two receptors. Molecular analysis of the patient tumours may also aid in identifying biomarkers of response.

In conclusion, neither IGF1R expression nor IGF1R/IR phosphorylation predicted response to trastuzumab in this cohort of early stage HER2-positive breast cancer patients. However, co-targeting the HER2 and IGF1R signalling pathways in HER2-positive breast cancer may prove beneficial if appropriate predictive biomarkers can be identified.

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