Increased signalling of EGFR and IGF1R, and deregulation of PTEN/PI3K/Akt pathway are related to trastuzumab resistance in HER2 breast carcinomas

A Gallardo1, E Lerma*,1, D Escuin2, A Tibau3, J Muñoz1, B Ojeda3, A Barnadas3, E Adrover4, L Sánchez-Tejada5, D Giner5, F Ortiz-Martínez5 and G Peiró5

1Department of Pathology, Hospital de la Santa Creu i Sant Pau, Autonomous University of Barcelona, Avda. Sant Antoni Mª Claret 167, 08025, Barcelona, Spain; 2Department of Clinical Oncology, Institut de Recerca, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain; 3Department of Clinical Oncology, Hospital General Universitario, Alicante, Spain; 4Department of Clinical Oncology, Hospital de la Santa Creu i Sant Pau, Autonomous University of Barcelona, Barcelona, Spain; 5Department of Clinical Oncology, Hospital General Universitario, Alicante, Spain

BACKGROUND: Trastuzumab resistance hampers its well-known efficacy to control HER2-positive breast cancer. The involvement of PI3K/Akt pathway in this mechanism is still not definitively confirmed.

METHODS: We selected 155 patients treated with trastuzumab after development of metastasis or as adjuvant/neoadjuvant therapy. We performed immunohistochemistry for HER2, ER/PR, epidermal growth factor 1-receptor (EGFR), α-insulin-like growth factor 1-receptor (IGF1R), phosphatase and tensin homologue (PTEN), p110α, pAkt, pBad, pmTOR, pMAPK, MUC1, Ki67, p53 and p27; mutational analysis of PIK3CA and PTEN, and PTEN promoter hypermethylation.

RESULTS: We found 46% ER/PR-positive tumours, overexpression of EGFR (15%), α-IGF1R (25%), p110α (19%), pAkt (28%), pBad (22%), pmTOR (23%), pMAPK (24%), MUC1 (80%), PTEN loss (20%), and PTEN promoter hypermethylation (20%). PIK3CA and PTEN mutations were detected in 17% and 26% tumours, respectively. Patients receiving adjuvant trastuzumab with α-IGF1R or pBad overexpressing tumours presented shorter progression-free survival (PFS) (all P < 0.043). Also, p110α and mTOR overexpression, liver and brain relapses implied poor overall survival (OS) (all P < 0.041). In patients with metastatic disease, decreased PFS correlated with p110α expression (P = 0.024), whereas for OS the presence of vascular invasion and EGFR expression (P < 0.019; Cox analysis).

CONCLUSION: Our results support that trastuzumab resistance mechanisms are related with deregulation of PTEN/PI3K/Akt/mTOR pathway, and/or EGFR and IGF1R overexpression in a subset of HER2-positive breast carcinomas.

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Keywords: breast cancer; HER2; EGFR; IGF1R; PTEN/PI3K/Akt pathway; trastuzumab resistance

Breast cancer (BC) is one of the most frequent malignancies in women (Jemal et al, 2008). HER2 overexpressing and/or gene amplified tumours represent approximately 25% of all BC, and they are associated with an aggressive phenotype, metastases, resistance to chemotherapy (CT), and poor prognosis (Slamon et al, 1987, 1989; Peiro et al, 2007; Nguyen et al, 2008). Nevertheless, the outcome has changed dramatically with the introduction of trastuzumab, a humanised monoclonal antibody that targets the HER2 extracellular domain (Murphy and Modi, 2009). It is very effective in combination with CT for the treatment of early stages (Viani et al, 2007) or metastatic BC (Pegram et al, 2004; Bruuskv et al, 2005), and even as a single-agent for the later group (Vogel et al, 2002), showing in both groups of patients a substantial decrease in cancer recurrence and mortality (Slamon et al, 2001; Piccart-Gebhart et al, 2005; Joensuu et al, 2006; Untch et al, 2008). Despite its demonstrated clinical benefit, about 30 – 50% of patients do not respond, and those with metastasis that achieved an initial response to trastuzumab-based regimens will develop drug resistance.

Currently, in clinical practice there are not conclusive biomarkers that allow the selection of patients who will respond to trastuzumab and the exact molecular mechanisms are still not well defined. Several growth factor receptors and signalling molecules have been proposed to be responsible for trastuzumab resistance, such as downregulation of the surface HER2 protein by endocytosis and degradation (Austin et al, 2004), p27 downregulation (Lane et al, 2001; Nahta et al, 2004), activation of insulin-like growth factor 1-receptor (IGF1R) (Lu et al, 2001; Nahta et al, 2005), interaction between HER2 and epidermal growth factor 1-receptor (EGFR) (Diermeier et al, 2005), phosphatase and tensin homologue (PTEN) loss (Nagata et al, 2004), phosphoinositide 3-kinase (PI3K)/Akt activation (Esteva et al, 2011; Razis et al, 2011), MUC1 (Fessler et al, 2009) and MUC4 upregulation (Nagy et al, 2005), and the crosstalk with the ER signalling pathway (Slamon et al, 2001). More recently, the non-receptor tyrosine kinase c-SRC (SRC) has been suggested as a potential key modulator of trastuzumab response (Zhang et al, 2011).

Therefore, the aim of our study was to evaluate the relevance of alterations in the PI3K/Akt/mTOR and Ras/mitogen-activated
protein kinase (MAPK) signalling pathways, given their role in cell cycle progression. We performed an extensive immunohistochemical and molecular analysis of several biological markers related with these pathways, in a series of patients with HER2-positive BC in stage I-IV, to determine their prognostic relevance, and as a result, their potential involvement in the mechanisms of response to trastuzumab.

PATIENTS AND METHODS

Tumour samples and patients’ follow-up

The study was conducted according to the Declaration of Helsinki principles, with approval from the local ethics committees. A total of 155 tumour samples from HER2-positive patients were retrospectively collected from the Department of Pathology of the Hospital de la Santa Creu i Sant Pau (n = 103) and University General Hospital of Alicante (n = 52). Patients were staged according to the WHO system, and tumours were histologically graded according to Elston and Ellis method. After pathological diagnosis, patients were treated according to standard protocols. All patients received trastuzumab for the treatment of metastatic disease (n = 75) after failure of conventional CT with anthracyclines and/or taxanes, or for early stages either adjuvant (n = 40) or neoadjuvant (n = 27) therapy. In 13 patients the type of treatment was unknown. Median follow-up was 5.3 years (range 0.17–31 years).

We considered response or non-resistance to trastuzumab treatment when no progression of stable disease occurred. Progression-free survival was defined as the length of time after treatment during which a patient survived with no signs of the disease, and OS as the time to the patients’ death or last follow-up.

Immunohistochemistry

Tissue microarrays were prepared from paraffin-embedded tissue taken from three representative tumour areas. Sections were stained using the Envision method (Dako, Glostrup, Denmark). HER2 protein and EGFR protein determinations were performed using HercepTest and EGFR pharmaDx (Dako; Glostrup, Denmark), respectively. Antibodies, dilutions, antigen retrieval methods, and suppliers are listed in Table 1. ER/PR and HER2 were evaluated by standard protocols. The EGFR expression was considered positive when complete membrane staining is >10% of tumour cells. The PTEN, pAkt, pBAD, p110α, mTOR, z-IGF1R, MUC1, and pMAPK (cytoplasm) scores were calculated by multiplying the percentage of labelled cells by the staining intensity (range 0–300). Loss of PTEN was considered for cutoff scores <75; and overexpression of p110α, MUC1, pMAPK, p27 and pAkt were considered for scores ≥150. Positive z-IGF1R and mTOR were considered for scores ≥220 and ≥30, respectively. The percentage of stained nuclei was evaluated independently of the intensity for Ki67 (cutoff 20%) and p53 (cutoff 10%). Consensus between three pathologists (AG, EL, and GP) was done for the immunohistochemical results.

Mutational analysis of PIK3CA

Genomic DNA was extracted from frozen tumour or paraffin-embedded tissues and mutational analysis of PIK3CA was performed by PCR and direct sequencing using primers for exons 9 and 20 as previously described (Samuels et al, 2004).

Phosphatase and tensin homologue mutation and promoter hypermethylation

Mutational analysis was performed using previously reported PCR conditions and primers for exons 3, 5, 7, and 8 (Bussaglia et al, 2000). Methylation-specific PCR was used to assay CpG island methylation status of the PTEN promoter gene using the Methylenzyme One-Step DNA Modification kit (Epigenetics, Brooklyn, NY, USA). Three primers sets were used for the PCR as previously reported (Soria et al, 2002).

In situ hybridisation analysis

HER2 gene status was confirmed by fluorescence in situ hybridisation (Dako pharmaDx) or chromogenic in situ hybridisation (Spot light; Zymed, Paisley, UK) in equivocal cases.

Statistical analyses

They were performed with the SPSS/win 17.0 statistical software package (SPSS, Chicago, IL, USA). Qualitative variables were compared with the X2/Fisher tests. A receiver operating characteristic curve and area under the curve were generated to determine a cutoff value of the expression of several biomarkers and the potential clinical utility to predict prognosis. The Kaplan–Meier method and the Cox regression model were used to estimate survival. P-values < 0.05 were considered statistically significant.

RESULTS

Clinicopathological data

The clinicopathological data is summarised in Table 2. Patients were classified into two groups: group A (n = 75) included patients where trastuzumab was included for treatment of metastatic disease and group B (n = 67 patients) those with trastuzumab in the adjuvant/neoadjuvant setting. Median age was 55 years (range 31–92 years) and median tumour size was 2.5 cm (range 1–20 cm). Histological grade 1 was seen in 7 (5%) cases, grade 2 in 50 (32%), grade 3 in 75 (55%) and grade 4 in 15 (10%) cases.

Table 1 Panel of antibodies for the immunohistochemical analysis

| Antibody | Clone | Dilution | Supplier | Pretreatment |
|----------|-------|----------|----------|-------------|
| ER       | 6F11  | 1:40     | Novocastra (Newcastle, UK) | Citrate buffer pH 6. Autoclave, 8 min |
| PR       | 16    | 1:200    | Novocastra (Newcastle, UK) | Citrate buffer pH 6. Autoclave, 8 min |
| z-IGF1R | 24–31 | 1:200    | Neomarkers (Fremont, CA, USA) | Citrate buffer pH 6. Autoclave, 8 min |
| PTEN     | 6H21 | 1:50     | Cascade Biosciences (Winchester, MA, USA) | Citrate buffer pH 6. Autoclave, 8 min |
| p110α    | Rabbit polyclonal | 1:25 | Cell Signaling (Beverly, MA, USA) | EDTA buffer pH 8. Autoclave, 8 min |
| pAkt     | Rabbit monoclonal | 1:50 | Cell Signaling (Beverly, MA, USA) | EDTA buffer pH 8. Autoclave, 8 min |
| pBAD     | Sc-12969-R | 1:40 | Santa Cruz (Santa Cruz, CA, USA) | EDTA buffer pH 8. Autoclave, 8 min |
| mTOR     | Rabbit polyclonal | 1:50 | Cell Signaling (Beverly, MA, USA) | EDTA buffer pH 8. Autoclave, 8 min |
| MUC1     | BC-2 | 1:40     | Signet (Dedham, MA, USA) | EDTA buffer pH 8. Autoclave, 8 min |
| pMAPK    | Rabbit IgG monoclonal | 1:100 | Dako (Carpinteria, CA, USA) | Citrate buffer pH 6. Autoclave, 8 min |
| Ki67     | MIB-1 | Prediluted | Dako (Carpinteria, CA, USA) | Citrate buffer pH 6. Autoclave, 8 min |
| p53      | DO-7  | Prediluted | Dako (Carpinteria, CA, USA) | Citrate buffer pH 6. Autoclave, 8 min |
| p27      | SX53G8 | 1:50 | Dako (Carpinteria, CA, USA) | EDTA buffer pH 8. Autoclave, 8 min |
and grade 3 in 98 (63%) tumours. Vascular invasion was found in 32% (47 of 145) cases. Axillary lymph node dissection was performed in 135 patients, being positive in 89 cases (66%). Tumour stage was IA in 17 (11%) patients, IIA in 29 (18.8%), IIB in 15 (9.7%), IIIA in 42 (27%), IIIB in 18 (11.6%), IIIC in 12 (7.7%), IV in 13 (8.4%), and was unknown in 9 (5.8%) patients. A total of 11 patients were lost in the follow-up, and among those remaining, 56 (39%) were alive with no evidence of disease, 31 (21.5%) alive with disease, and 57 (39.5%) died of the disease (DOD).

Tumour molecular features

Table 3 includes the relationship between clinicopathological, immunohistochemical and molecular data for all tumours.

**Table 2** Summary of the main clinicopathological data

| All cases (n = 155) | Trastuzumab in the metastatic disease (n = 75) | Trastuzumab in the first-line treatment (n = 67) |
|---------------------|---------------------------------------------|---------------------------------------------|
| Age (median and range) | 55 years (31 – 92 years) | 59 years (31 – 92 years) | 54 years (33 – 88 years) |
| Tumour size (median and range) | 2.5 cm (1 – 20 cm) | 2.8 cm (1 – 11 cm) | 2.4 cm (4 – 20 cm) |
| BC subgroups | | | |
| HER2+/HR+ | 67 | 35 | 29 |
| HER2+/HR- | 78 | 33 | 37 |
| Unknown | 10 | 7 | 1 |
| Lymph node status | | | |
| Negative | 46 | 20 | 24 |
| Positive | 89 | 47 | 36 |
| Unknown | 20 | 8 | 7 |
| Stage | | | |
| IA | 17 | 8 | 8 |
| IIA | 29 | 10 | 17 |
| IIB | 15 | 8 | 6 |
| IIIA | 42 | 22 | 17 |
| IIIB | 18 | 13 | 5 |
| IIC | 12 | 9 | 3 |
| IV | 13 | 3 | 7 |
| Unknown | 9 | 2 | 4 |
| Histological grade | | | |
| 1 | 7 | 1 | 4 |
| 2 | 50 | 24 | 23 |
| 3 | 98 | 50 | 40 |
| DCIS <25% | 25 | 12 | 12 |
| >25% | 22 | 9 | 13 |
| Follow-up | | | |
| NED | 56 | 5 | 50 |
| AWD | 31 | 21 | 10 |
| DOO | 57 | 49 | 7 |
| LFU | 11 | 0 | 0 |

**Table 3** Statistical correlations between clinicopathological, immunohistochemical and molecular data for all tumours

| | Histological grade | Ductal form | Nuclear grade | Mitosis | Lymph node + | Vascular invasion |
|---------------------|---------------------|-------------|--------------|----------|--------------|------------------|
| HR+ | 0.080* | 0.024* | NS | 0.091* | NS | NS |
| EGFR+ | 0.061 | 0.083 | NS | 0.013 | 0.088 | NS |
| zIGF1R+ | 0.001 | NS | 0.07 | 0.004 | NS | 0.005 |
| PTEN loss | 0.065 | NS | NS | NS | 0.047 |
| PIK3CA mut | NS | NS | 0.043 | NS | NS | NS |
| pAkt+ | NS | NS | NS | NS | NS | NS |
| pBad+ | 0.001 | NS | 0.008 | 0.002 | NS | 0.006 |
| mTOR+ | NS | NS | 0.034 | NS | 0.12 | NS |
| MAPK+ | 0.029a | NS | NS | NS | NS | NS |
| Ki67 > 20% | 0.087 | NS | 0.021 | NS | 0.082 |
| p53 > 10% | NS | NS | 0.009 | 0.076 | NS | NS |
| p27+(nuclear) | NS | NS | NS | NS | NS | NS |

**Figure 1** Immunohistochemical expression of EGFR, zIGF1R, p110a, pAKT, pBad, and loss of PTEN in HER2-positive breast carcinomas.

Staining of z-IGF1R was strong and diffuse (overexpression) in 25% tumours (34/138), in association with high grade (P = 0.001), high mitotic index (P = 0.004), and vascular invasion (P = 0.005).

Biomarkers associated with the PI3K/Akt/mTOR and MAPK signalling pathways Phosphatase and tensin homologue loss was found in 20% of the tumours (30/149), PTEN promoter hypermethylation in 20% (22/110) and mutations in 26% (8/30). Phosphatase and tensin homologue loss was associated with vascular invasion (P = 0.047) and higher grades (P = 0.065), but
neither association with PTEN mutation nor hypermethylation was found.

p110α (PI3K catalytic subunit) overexpression was present in 19% of the tumours (24/125), and PIK3CA somatic missense mutations were identified in 17% (24/142) in exon 20 (nucleotide A3140G, amino acid H1047R) in 15% of the tumours (21/142), whereas mutations in the helical domain of exon 9 (nucleotide G1635C, amino acid H1047R) were identified in 17% (24/142): in exon 20 (nucleotide A3140G, amino acid H1047R) overexpression was found in 28% of the tumours (40/143) and phosphorylated (inactive) Bad in 22% (30/139) in association with high nuclear grade (P = 0.008) and histological grades (P = 0.001), elevated mitotic index (P = 0.002), and vascular invasion (P = 0.006). mTOR overexpression was detected in 23% (33/142) of the tumours, predominantly with high nuclear grade tumours (P = 0.034), and in association with p-IGF1R (47%; P < 0.000), p110α (64%; P = 0.028) and pBad (65%; P = 0.027).

Strong pMAPK expression in 24% (22/93) of the tumours predominated in those of low grade (P = 0.029); MUC1 was overexpressed in 80% of the analysed tumours (77/96) but without significant association with clinicopathological features.

**Cell proliferation and apoptosis markers**  
Ki67 > 20% was seen in 51% of the tumours (73/144), related with high mitotic index (P = 0.021). p53 overexpression was found in 30% of the tumours (42/139) associated with HR-negative status (P = 0.022) and high nuclear grade (P = 0.009). Only 17% (16/94) of the cases showed p27 nuclear expression but unrelated with any clinical-pathological factors.

**Relationship between biomarkers and recurrence**

In all, 61% of the patients developed distant metastases, which were located in the liver (35%), bone (35%), lung (27%), lymph nodes (21%), pleura (18%), central nervous system (CNS; 16%), and skin (14%). Patients with HR-positive tumours presented more frequently bone metastases (P = 0.008) compared with those with HR-negative status. In contrast, tumours with p-IGF1R overexpression rarely metastasised to the liver (P = 0.009), lung (P = 0.002), bone (P = 0.031) or lymph nodes (P = 0.007). Patients with p110α-positive tumours developed more frequently CNS metastases (P = 0.029). The remaining proteins studied here showed a trend or were not associated with any specific site of dissemination (see Table 4).

**Survival analyses**

In order to perform the survival analysis in similar patients groups, we excluded those that received neoadjuvant CT or stage IV. Therefore, 51 patients remained in group A and 38 patients in group B. Supplementary Figures 1 and 2 include the Kaplan–Meier curves for both groups. Table 5 shows the results of the multivariate analysis.

**Metastatic BC (group A)**  
A total of 47 patients (92%, 47/51) had tumour recurrence with a median PFS of 2.6 years (range 1.01 to 11.64 years) and 65% (33/51) of the patients DOD with a median OS of 7.5 years (range 0.17 to 21 years).

Univariate analysis (Kaplan–Meier; log rank test) showed that shorter PFS was associated with vascular invasion (P = 0.042), mutated PTEN (P = 0.045), EGFR (P = 0.026), p110α (P = 0.004), pAkt overexpression (P = 0.016), and CNS metastases (P = 0.002). Poor OS correlated with positive lymph node status (P = 0.013), EGFR (P = 0.006), p110α (P = 0.079), pAkt overexpression (P = 0.042), tumour stage (P = 0.003), and tumour relapses in the liver (P = 0.059) or in CNS (P = 0.005).

Multivariate analyses for PFS revealed that only the presence of metastases to the CNS (P = 0.020, HR 3.59, CI 1.23–10.51) and p110α overexpression (P = 0.024, HR 2.75, CI 1.14–6.49) emerged

### Table 4  Statistical significance according to metastatic site for all patients. (Note: EGFR, p53, p27, and MAPK expression levels were unrelated with metastases)

| Variables | Liver | Bone | CNS | Skin and soft tissue | Lung | Pleura | Lymph nodes |
|-----------|-------|------|-----|---------------------|------|--------|-------------|
| HR+       | NS    | 0.008| NS  | 0.069*              | NS   | NS     | NS          |
| ER+       | NS    | 0.004| NS  | 0.082               | NS   | NS     | NS          |
| PR+       | NS    | 0.044| NS  | 0.090               | NS   | NS     | NS          |
| aEPGR+    | 0.009*| 0.031*| NS  | NS                  | NS   | 0.002*| 0.007*      |
| PTEN      | - Loss expr | NS | NS  | 0.058               | NS   | NS     | NS          |
|           | - Mutat | NS  | NS  | 0.065               | NS   | 0.099  | NS          |
| p110α     | NS    | NS   | NS  | 0.0029              | NS   | NS     | NS          |
| PIK3CA    | - Mutat | NS  | NS  | NS                  | NS   | 0.087  | NS          |
| pAkt+     | NS    | 0.085| NS  | NS                  | NS   | NS     | NS          |
| mTOR+     | 0.069 | NS   | NS  | NS                  | NS   | NS     | NS          |
| Ki67 >20% | 0.011 | 0.011| 0.037| NS                  | NS   | 0.049  | 0.096       |
| pBad+     | 0.068 | NS   | NS  | NS                  | NS   | NS     | NS          |

**Abbreviations:** CNS = central nervous system; ER = oestrogen receptor; HR = hormonal receptors; IHC = immunohistochemistry; mutat = mutations; NS = non-significant; PR = progesterone receptor. *Inverse relationship.

### Table 5  Multivariate analysis of histological and biological factors for patients with trastuzumab treatment in the metastatic disease (group A) (Cox model)

| Variables | ß Hazard ratio | 95% CI | P-value |
|-----------|----------------|--------|---------|
| Disease-free survival | | | |
| CNS metastasis | 1.128 | 3.59 | 1.23–10.51 | 0.020 |
| p110α | 1.269 | 2.75 | 1.14–6.49 | 0.024 |
| Overall survival | | | |
| Vascular invasion | 1.17 | 3.36 | 1.22–8.94 | 0.015 |
| CNS metastasis | 1.406 | 4.22 | 1.44–12.38 | 0.009 |
| EGFR | 1.630 | 5.25 | 1.32–20.92 | 0.019 |

**Abbreviations:** CNS = central nervous system; EGFR = epidermal growth factor 1-receptor.

TRASTUZUMAB RESISTANCE IN HER2 BREAST CARCINOMAS

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as significant predictors of relapse. Worse OS was seen for vascular invasion (P = 0.015, HR 3.36; CI 1.22–8.94), EGFR expression (P = 0.019, HR 5.25; CI 1.32–20.92), and metastases to the CNS (P = 0.009, HR 4.22; CI 1.44–12.38) (Cox regression model).

**Early-stage BC (group B)** Only 11% (4/38) of the patients had tumour recurrence and 5% died from the tumour. These events might be, however, related to the short follow-up of the majority of the patients (median 2.82 years). Median PFS was 2.81 years (range 1.00–8.28 years) and for OS was 2.82 years (range 1.00–8.42 years).

Shorter PFS was associated with z-IGF1R (P = 0.028), pBad overexpression (P = 0.003), and tumour recurrence in the liver (P = 0.003) or the bone (P = 0.001). Poor OS correlated with high tumour grade (P < 0.000), overexpression of p110z (P = 0.041) and mTOR (77 vs 100% in negative cases, P = 0.006), and tumour recurrence in the liver (P = 0.009) and CNS (P = 0.011) (Kaplan–Meier; log rank test). Nevertheless, the multivariate analysis (Cox regression) showed that these results did not reach any statistically significance, probably due to the small number of events in this group.

**DISCUSSION**

In the current study, we performed an extensive immunohistochemical and molecular analysis of biological markers related with the PI3K/Akt/mTOR and Ras/MAPK signalling pathways in a series of HER2-positive BC patients who received trastuzumab for metastatic disease or as first-line therapy in earlier stages. We found that patients with primary tumours showing alterations in EGF R and PTEN/PI3K/Akt had shorter PFS and OS despite trastuzumab treatment when given at advanced stage (metastatic disease), supporting their role in the mechanisms of response. Our results in the group of patients in earlier stages who received trastuzumab as adjuvant/neoadjuvant therapy demonstrated that those having tumours with IGF1R overexpression and inactive Bad had shorter PFS. Poorer OS was seen in patients who developed metastatic disease especially in the brain and liver, and with p110z and mTOR overexpressing tumours. Nevertheless, none of the factors had an independent prognostic value, probably related with the small number of events and short follow-up of this group.

PI3K/Akt signalling is one of the most critical cancer-promoting pathways through upregulation of growth factor receptors (EGFR, IGF1R, HER2, etc) or PTEN inactivation (Lu et al, 1999) and recently considered a major determinant of trastuzumab resistance (Nagata et al, 2004; Berns et al, 2007; Esteva et al, 2011; Razis et al, 2011). HER2 and EGFR coexpression has a considerable inhibitory effect on this drug (Diermeier et al, 2005) and are associated with poor prognostic factors such as high grade, negative HR status, and vascular invasion (Abd El-Rehim et al, 2004). In agreement with these findings, we found coexpression in 15%, which in turn was related with PIK3CA mutations. Insulin-like growth factor 1-receptor has an important role in growth and invasiveness of BC (Peiro et al, 2009, 2011) and recently has also been involved in trastuzumab resistance (Lu et al, 2001; Nahta et al, 2005; Harris et al, 2008). Moreover, we found IGF1R overexpression in 15% of the tumours, especially in those of early stage patients who developed recurrences. Of note, there is considerable evidence that both IGF1R and EGFR crosstalk in BC cells and their coactivation occurs in approximately 25% of BC, related with poor outcome (Harris et al, 2001, 2007; Lu et al, 2001; Abd El-Rehim et al, 2004). Therefore, it would be expected that those patients would be more likely to be resistant to trastuzumab.

**PTEN** encodes a protein that inhibits activation of the PI3K/Akt/mTOR signalling pathway (Panigrahi et al, 2004). The PTEN inactivation may be related with gene mutations (~5% of sporadic BC) (Vanhaesebroeck and Alessi, 2000) or promoter hypermethylation (20%) (Saal et al, 2003), resulting in PTEN loss that occurs in about half of the tumours (Nagata et al, 2004; Lerma et al, 2008; Esteva et al, 2011; Razis et al, 2011). Prior experimental studies with SKBR3 and BT474 BC cells and in breast tumour xenografts demonstrated that PTEN reduction confers resistance to trastuzumab’s antitumour function, and this data was subsequently confirmed in a group of patients (Nagata et al, 2004). In the current study, PTEN loss or promoter hypermethylation were observed in both in 20% of the tumours but without association with patient’s survival, despite their correlation with other adverse clinicopathological data, such as vascular invasion and lymph node metastases. Nevertheless, tumours with PTEN mutations (26%) recurred more frequently in patients with metastatic disease, supporting its contribution to trastuzumab resistance.

**PI3K/Akt pathway** activation blocks apoptosis and promotes cell proliferation through the PI3K/Akt activation, with different downstream effectors (Stiemke-Hale et al, 2008; Di Cosimo and Baselga, 2008; Nahta and O’Regan, 2011; Margariti et al, 2011). PIK3CA activating mutations, clustered in exons 9 (helical domain) and 20 (kinase domain) have been reported in 18–40% BC, occasionally associated with HER2 phenotype (Stiemke-Hale et al, 2008) and tumour recurrence (Razis et al, 2011). We found PIK3CA mutations in 17% of the tumours, unrelated with trastuzumab clinical benefit. In contrast, p110z overexpression (19%) had an independent poor prognostic value for progression in patients with advanced stage. Moreover, active Akt in 28% of our tumours, correlated with recurrence and poor patients’ survival, supporting that activation of this pathway contributes to tumour growth and therefore to trastuzumab resistance. Further, inactive Bad seen in 22% of the tumours in association with adverse prognostic parameters, such as high tumour grade, high mitotic index and vascular invasion, predicted shorter survival as a result of non-response, in early stage patients. In partial agreement with our data, Esteva et al (2011), in a previous series of 137 metastatic BC, found that PI3K pathway activation (defined as PI3K loss and/or PIK3CA mutation) significantly contributed to worse response to trastuzumab and shorter OS. Moreover, pAkt and PTEN status combination showed more power than PTEN loss alone.

mTOR is a key regulator of multiple cell stimuli integrating growth factor and cytokine signals. In vitro studies and recent clinical data have confirmed a relationship between mTOR and HER2 (Morrow et al, 2011) as well as its role in trastuzumab resistance (Nahta and O’Regan, 2011). In our series, 32% of patients presented concomitant increased mTOR, and these patients had lymph-node metastasis. Of note, we found that mTOR modulation by PI3K/Akt-dependent mechanisms reflected by its positive correlation with p110z and Bad is influenced by IGF1R. Further, supporting its involvement in the mechanisms of trastuzumab responsiveness, all our patients from the group B and negative mTOR tumours were alive at the last follow-up compared with only 77% for those with positive tumours. This is of interest as preclinical models have shown that dual inhibition of both IGF1R – with either monoclonal antibodies or tyrosine kinase inhibitors – and mTOR results in a superior antiproliferative effect over each single strategy, and this combination is now under evaluation in phase I/II trials in patients with BC (Di Cosimo and Baselga, 2009). Nevertheless, recent data support the assertion that trastuzumab has less effect on this cell cycle kinetics pathway (Dave et al, 2011), and therefore, not relevant in the development of resistance, in line with our study.
Using a full-length MUC1 antibody, we found expression in 80% of the tumours, with neither correlation with prognostic indicators nor survival. Recent studies have now shown it is not related to tumour growth but it is present in newly differentiated stem cells. However, the cleaved form of the MUC1 protein (MUC1*) has growth factor receptor-like activity on tumour cells and is detected in populations of pluripotent stem cells (Hikita et al, 2008; Mahanta et al, 2008). Fessler et al (2009) showed upregulation of MUC1* in HER2-amplified/trastuzumab-resistant BC cells. Further treatment with MUC1* antagonists in addition to trastuzumab reversed that resistance (Fessler et al, 2009). Therefore, further studies on MUC1* are needed to confirm the previous data in clinical series.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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In summary, we found in about one-forth of HER2 tumours at least one molecular alteration in the PI3K pathway and/or its upstream or downstream effectors. Our data support the complex interactions between EGFR, IGF1R, and the PTEN/PI3K/Akt/Bad and mTOR signalling pathway, which in turn are potentially related with the mechanisms of trastuzumab response. Nevertheless, some of these biomarkers need to be further validated in larger series and introduced into the clinical practice to carefully select patients on the basis of tumour molecular alterations. This is of relevance as novel combinations for targeting simultaneously several factors might suggest another strategy to overcome trastuzumab resistance and enhance response rates.
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