Src, a potential target for overcoming trastuzumab resistance in HER2-positive breast carcinoma

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Background: Src is a non-receptor tyrosine kinase involved in signalling and crosstalk between growth-promoting pathways. We aim to investigate the relationship of active Src in response to trastuzumab of HER2-positive breast carcinomas.

Methods: We selected 278 HER2-positive breast cancer patients with (n = 154) and without (n = 124) trastuzumab treatment. We performed immunohistochemistry on paraffin-embedded tissue microarrays of active Src and several proteins involved in the PI3K/Akt/mTOR pathway, PIK3CA mutational analysis and in vitro studies (SKBR3 and BT474 cancer cells). The results were correlated with clinicopathological factors and patients’ outcome.

Results: Increased pSrc-Y416 was demonstrated in trastuzumab-resistant cells and in 37.8% of tumours that correlated positively with tumour size, necrosis, mitosis, metastasis to the central nervous system, p53 overexpression and MAPK activation but inversely with EGFR and p27. Univariate analyses showed an association of increased active Src with shorter survival in patients at early stage with HER2/hormone receptor-negative tumours treated with trastuzumab.

Conclusions: Src activation participates in trastuzumab mechanisms of resistance and indicates poor prognosis, mainly in HER2/ hormone receptor-negative breast cancer. Therefore, blocking this axis may be beneficial in those patients.
Src in HER2-positive breast carcinoma

Tumour samples and patients’ follow-up. The current retrospective study is based on a cohort of 278 unselected consecutive HER2-positive breast carcinomas. Patients were classified into three groups depending on the modality of treatment as follows: group A (n = 124) included those who received chemotherapy (CT) and no trastuzumab; group B (n = 76) included patients who received trastuzumab for the treatment of metastatic disease (first-line therapy) after failure of the conventional CT with anthracyclines and/or taxanes, and group C (n = 65) included those who received trastuzumab combined with CT with anthracyclines and/or taxanes for early stages in the adjuvant/neoadjuvant setting. The characteristics of trastuzumab-treated group have been described in detail previously (Gallardo et al., 2012). Treatment was given in a neoadjuvant setting in 4 patients in group A, 22 patients in group B and 21 in group C. In 13 patients, the type of treatment was unknown. Moreover, patients with ER and/or PR-positive tumours received tamoxifen or aromatase inhibitors for 2–5 years. Of note, in group A it was 8.28 years (n = 46; range 1.34–20.9 years) and in group C it was 3.6 years (n = 36; range 1.13–8.12 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 overall survival (OS) as the time to the patients’ death or last follow-up.

Immunohistochemistry (IHC). It was carried out on serial TMA sections using the EnVision Flex detection system (Dako/Agilent Technologies, Carpinteria, CA, USA). Antibodies, suppliers, dilutions, conditions and cutoffs are listed in Table 1. Staining results were semiquantitatively scored according to the percentage of positive cells and intensity (0 to 3+) (Histo-score 0–300). Src activation (Src phosphorylated at Tyr416) status was studied in all tumours and correlated with several Src-dependent biomarkers and patients’ outcome. Tumours with pSrc-Y416 membrane +/− cytoplasm staining of at least 5% of cells with moderate/strong (2–3+) intensity were considered positive. As negative controls, staining was carried out in the absence of the primary antibodies.

In situ hybridisation (ISH) analysis. HER2 gene status was confirmed by chromogenic ISH (Spot light; Zymed, San Francisco, CA, USA) or fluorescence ISH (Dako pharmDx) in non-definitive cases (2+ and <10% 3+ cells) (Peiro et al, 2007).

Mutational analysis of PIK3CA. Genomic DNA was extracted from frozen or paraffin-embedded tumours and mutational analysis of PIK3CA was performed by PCR and direct sequencing using primers for exons 9 and 20, as previously described (Gallardo et al., 2012).

Cell culture. BT747 (HER2/ER-positive) and the SKBR3 (HER-positive/ER-negative) human breast cancer cells were cultured in DMEM/Ham’s F-12 (1:1) (PAA, Cöble, Germany), supplemented with 10% fetal bovine serum (PAA), penicillin (100 U ml−1) and streptomycin (100 μg/ml−1) and maintained at 37 °C in a humidified atmosphere of 5% CO2. Trastuzumab-resistant BT747 and SKBR3 cells were developed by adding freshly prepared trastuzumab (4 μg/ml−1 or 8 μg/ml−1) twice a week for 4 months; cells were passaged when 70–80% confluence was reached. The resistance to trastuzumab was tested by dose–response studies in parental and resistant cells and western blotting analysis, as described below.

Dose–response studies. SKBR3 and BT747 parental and trastuzumab-resistant cells were seeded in 96-well plates at a density of 1 or 2 × 103 cells per well, respectively. After 24 h, cell media was replaced and newly added with trastuzumab at different concentrations or DMSO (vehicle), using four wells per concentration. After 5 days, MTT reagent was added and incubated for 3 h at 37 °C in a humidified atmosphere of 5% CO2. Cell media was then replaced with 200 μl of DMSO, and after 30 min, optical density was measured at 570 nm in a microplate reader. The experiment was performed in triplicate at different times.

Western blotting analysis. Protein lysates were obtained from parental and trastuzumab-resistant cells using a lysis buffer composition of 20 mM Tris at pH 7.0, 1% Triton-X 100, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 2 mM DTT and protease

Materials and methods

Tumour samples and patients’ follow-up. The current retrospective study is based on a cohort of 278 unselected consecutive HER2-positive breast carcinomas. Patients were classified into three groups depending on the modality of treatment as follows: group A (n = 124) included those who received chemotherapy (CT) and no trastuzumab; group B (n = 76) included patients who received trastuzumab for the treatment of metastatic disease (first-line therapy) after failure of the conventional CT with anthracyclines and/or taxanes, and group C (n = 65) included those who received trastuzumab combined with CT with anthracyclines and/or taxanes for early stages in the adjuvant/neoadjuvant setting. The characteristics of trastuzumab-treated group have been described in detail previously (Gallardo et al., 2012). Treatment was given in a neoadjuvant setting in 4 patients in group A, 22 patients in group B and 21 in group C. In 13 patients, the type of treatment was unknown. Moreover, patients with ER and/or PR-positive tumours received tamoxifen or aromatase inhibitors for 2–5 years. Of note, trastuzumab untreated patients (group A) were diagnosed and treated before trastuzumab therapy was approved at both Institutions.

Tumours were collected from the Departments of Pathology of the University General Hospital of Alicante (n = 196) and Hospital de la Santa Creu i Sant Pau (n = 82), Barcelona, Spain. The study was conducted according to the Declaration of Helsinki principles. The Institutional Review Board at both Institutions approved the study and waived the requirement for informed consent in patients with the diagnosis before 2007.
inhibitor cocktail. Cell lysate was collected after centrifugation at 14,000 r.p.m., 10 min at 4 °C, separated on SDS–PAGE gel at 8% polyacrylamide and blotted onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked in 5% milk in PBS-Tween for 1 h and then probed with primary antibodies Monoclonal Mouse Anti-Human HER2-pY1248 (Dako, Glostrup, Denmark); Src (36D10) #2146 and Src-phosphorylated (p Src-Y416). In contrast, in patients treated with trastuzumab-based therapy, increased Src activation implied poorer survival in patients with PTEN protein loss or with p-mTOR.

**Statistical analyses.** The chi-square or Fisher’s tests were used to determine the distribution of the clinical-pathological, IHC and molecular characteristics. A receiver operating characteristic and trend with statistical software package (SPSS, Chicago, IL, USA). P-values <0.05 were considered statistically significant.

### RESULTS

The clinical-pathological data are summarised in Supplementary Table S1. Patients were classified into three groups as previously detailed. Median age was 56 years (range 30–92 years), and median tumour size was 23 mm (range 5–200 mm). Tumours were predominantly of grade 3 (171 out of 276; 61.5%) with necrosis (90 out of 171; 52.6%), no vascular invasion (203 out of 265; 76.6%), with positive lymph node status (146 out of 264; 55.3%) and presenting at early stage (IIA 28%). Thirteen patients were lost in the follow-up (5%), 141 (51%) were alive with no evidence of disease and 6 (2%) were dead of other causes.

Table 2 includes the relationship between pSrc-Y416 and clinicopathological data. Src activation (Figure 1) was seen in 37.8% (105 out of 278) of the tumours, in relation with larger tumour size (P=0.049), necrosis (P=0.043), high mitotic index (P=0.021) or metastasis to the central nervous system (P=0.009). Table 3 shows the correlations between Src activation and other molecular biomarkers. An inverse correlation was found between Src and EGFR expression (8.4%, P=0.006). Significant positive correlations were observed with pMAPK activation (25.6%; P<0.000), as well as p53 overexpression (43.4%, P=0.009) and p27 nuclear expression (74%; P=0.028), and as a trend with x-IGF1R (33%; P=0.16), p110α (22.9%; P=0.11) or pAkt (28.4%; P=0.2). However, no association was seen either with PTEN protein loss or with p-mTOR.

The expression level of inactive/non-phosphorylated Src did not change in BT474- or SKBR3-resistant cells when compared with the parental cell line. In contrast, it was higher for pSrc-Y416 in trastuzumab-resistant cells (Figure 2).

At last follow-up, 44% of patients had distant metastases, which were located in the liver (41%), bone (38%), lung (28%), lymph nodes (21%), pleura (18%) or CNS (18%). Increased pSrc-Y416 expression was found in tumours that metastasised to CNS (P=0.009). Inverse associations were found between liver metastases and EGFR (P=0.042) and bone metastases with x-IGF1R (P=0.010), p-mTOR (P=0.008) or Ki67 (P=0.012).

Table 4 shows the results of the univariate analysis (Kaplan-Meier; log-rank test) for the group without trastuzumab. No differences in survival were observed for the activated status of pSrc-Y416. In contrast, in patients treated with trastuzumab-based therapy, increased Src activation implied poorer survival in patients at early stage (OS P=0.034) and in those with metastatic disease, as a trend (OS P=0.148). Interestingly, further analysis

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**Table 1. Panel of antibodies for the immunohistochemical analysis**

| Antibody | Clone | Dilution | Supplier | Pretreatment | Incubation | Staining/cutoff |
|----------|-------|----------|----------|-------------|------------|----------------|
| HER2 (HercepTest) | Rabb Pol | 1:1 | Dako | Citrate buffer pH 9 | 30 min, RT | Membrane, ≥10% 3+ |
| ER-α | 6F11 | 1:40 | Dako | Citrate buffer pH 6 | 20 min, RT | Nuclear, ≥1% |
| PR | PgR36 | 1:200 | Dako | Citrate buffer pH 6 | 20 min, RT | Nuclear, ≥1% |
| Ki67 | MIB-1 | 1:1 | Dako | Citrate buffer pH 9 | 20 min, RT | Nuclear, ≥14% |
| pSrc (Y416) | Rabbit M1 | 1:50 | Cell Sig | Citrate buffer pH 9 | 4°C overnight | Membrane/cytoplas, HS ≥5% 2-3+ |
| EGFR (pharmaDx) | H11 | 1:1 | Dako | Citrate buffer pH 9 | 30 min, RT | Membrane, ≥10% 3+ |
| α-IGF1R | 24-31 | 1:200 | NeoM | Citrate buffer pH 9 | 30 min, RT | Membrane/cytoplas, HS ≥220 |
| PTEN | 6H2.1 | 1:50 | Dako | Citrate buffer pH 6 | 20 min, RT | Membrane/cytoplas, HS ≥75 |
| p110α | Rabbit Pol | 1:80 | Cell Sig | Citrate buffer pH 9 | 4°C overnight | Membrane/cytoplas, HS ≥150 |
| pAkt (Ser473) | 14-5 | 1:10 | Dako | Citrate buffer pH 9 | 20 min, RT | Membrane/cytoplas, HS ≥150 |
| pmTOR (Ser2448) | Rabbit Pol | 1:50 | Cell Sig | Citrate buffer pH 9 | 20 min, RT | Membrane/cytoplas, HS ≥30 |
| pMAPK (Thr202/Tyr204) | 20G11 | 1:100 | Cell Sig | Citrate buffer pH 9 | 30 min, RT | Membrane/cytoplas, HS ≥150 |
| p53 | DO-7 | 1:1 | Dako | Citrate buffer pH 9 | 20 min, RT | Nuclear, ≥20% |
| p27 | SX53G8 | 1:50 | Dako | Citrate buffer pH 9 | 30 min, RT | Nuclear, ≥75% |

**Abbreviations:** EGFR = epidermal growth factor receptor; ER = oestrogen receptor; HER2 = human epidermal growth factor receptor; HS = Histo-score; IGF1R = insulin-like growth factor 1-receptor; Membr/cytoplas= membrane/cytoplasm; Mn = monoclonal; Pol = polyclonal; PR = progesterone receptor.
showed that specifically in patients under adjuvant trastuzumab with HR-negative tumours, increased pSrc-Y416 correlated with worse DFS (71% vs 100%; \( P = 0.032 \)) and OS (71% vs 100%; \( P = 0.033 \)) (Figures 3A and B).

### DISCUSSION

Current research has increased substantially the understanding of the abnormalities involved in the mechanisms of trastuzumab resistance. However, there are no validated biomarkers of resistance to this therapy. Our studies focused on acquired trastuzumab-resistant cells, and a clinical series of HER2 breast cancer patients showed activated Src in breast cancer cells and in a significant proportion of human tumours whose patients had poorer prognosis. This is in line with previous in vitro and in vivo preclinical resistance model studies (Lu et al, 2003; Nagata et al, 2004; Wang et al, 2009; Liang et al, 2010; Boyer et al, 2012; Rexer et al, 2012), indicating that activation status of pSrc-Y416 is involved in the mechanisms of resistance to trastuzumab.

Src has a role in signalling and crosstalk between growth-promoting pathways (Yeatman, 2004). Activated Src expression has been reported in 18–39% of breast carcinomas (Chu et al, 2007; Schmitz et al, 2005). In agreement, we found that 37.8% of our tumours showed active Src, especially in those of larger size, with high proliferation index, necrosis, mitosis, metastasis to the CNS and p53 overexpression, all related with a more aggressive phenotype. Moreover, several molecular alterations were also frequent, such as overexpression of IGF1R, p110α/pAkt, MAPK and p27. In contrast, there were no differences in PTEN or mTOR status. Collectively, our results support that Src activation is associated with an IGF1R-dependent mechanism involving activation of the MAPK and PI3K/Akt pathways, as reported in other types of neoplasia (Michels et al, 2013). However, an inverted correlation of Src with EGFR and p27 loss and not association with mTOR makes probable that other than IGF1R-dependent pathways may mediate Src activation in breast carcinoma as well (Chu et al, 2007; Ishizawar et al, 2007). In fact, experimental data indicate that Src and PTEN may regulate each other to promote trastuzumab resistance. On the one hand, PTEN inactivation occurs by Src through phosphorylation on tyrosine (Lu et al, 2003) and on serine/threonine at the carboxyl terminal (S380/T382/T383) of PTEN. The latter phosphorylation leads to increased PTEN stability but loss of its function (Vazquez et al, 2000). On the other hand, a novel mechanism suggests that PTEN directly and specifically dephosphorylates Src-Y416 by its protein phosphatase activity (Zhang et al, 2011). In the current study, we found no correlation between PTEN and Src activation status. Of note, among our tumours with active Src, 85.3% had PTEN-preserved expression; therefore, it is plausible that in some cases PTEN was non-functional. However, this issue was out of the scope of our study as we analysed the expression levels but not the functional status or subcellular location (cytoplasm vs membrane).

Deregulation of the PI3K/Akt pathway has been associated with resistance to the HER2 inhibitors (Nagata et al, 2004; Esteva et al, 2010), and adverse outcome has been observed in patients with neoadjuvant- or adjuvant-trastuzumab treatment (Jensen et al, 2010; Gzikova et al, 2013). In the current study, the fact that p110α (PI3K catalytic subunit) and pAkt were overexpressed supports the influence of Src in this pathway in a subset of tumours.
Table 3. Relationship between pSrc-Y416 and molecular biomarkers

| Variables | All cases, n (%) | pSrcY416 ( + ), n (%) | pSrcY416 ( - ), n (%) | P-value |
|-----------|-----------------|-----------------------|----------------------|---------|
| Number    | 278 (100)       | 105 (37.8)            | 173 (62.2)           |         |
| HR status |                 |                       |                      |         |
| Positive  | 150 (54)        | 51 (45.6)             | 99 (57.6)            | 0.483   |
| Negative  | 118 (42.4)      | 43 (34.3)             | 56 (44.2)            |         |
| Unknown   | 10 (3.6)        | 11 (21.6)             | 11 (21.6)            |         |
| p53       |                 |                       |                      |         |
| <20%      | 174 (62.8)      | 56 (56.6)             | 118 (72.4)           | 0.009   |
| ≥20%      | 88 (31.7)       | 43 (43.4)             | 45 (27.6)            |         |
| Unknown   | 16 (5.8)        | 11 (4.3)              | 7 (4.4)              |         |
| Ki67      |                 |                       |                      |         |
| <14%      | 95 (34.2)       | 32 (33)               | 63 (40.1)            | 0.253   |
| ≥14%      | 159 (57.2)      | 65 (67)               | 94 (59.9)            |         |
| Unknown   | 24 (8.6)        | 8 (9.2)               | 2 (4.3)              |         |
| PTEN      |                 |                       |                      |         |
| Loss      | 47 (16.9)       | 15 (14.9)             | 32 (18.9)            | 0.373   |
| Present   | 224 (80.6)      | 87 (85.3)             | 137 (81.1)           |         |
| Unknown   | 7 (2.5)         | 1 (0.7)               | 1 (0.7)              |         |
| α-IGF1R   |                 |                       |                      |         |
| No overexpression | 191 (68.7) | 69 (67) | 122 (74.8) | 0.165 |
| Overexpression | 75 (27)     | 34 (33) | 41 (25.2) |         |
| Unknown   | 12 (4.3)        | 8 (7.9)              | 33 (20.5)           |         |
| EGFR      |                 |                       |                      |         |
| Negative  | 221 (79.5)      | 93 (92.1)             | 128 (79.5)           | 0.006   |
| Positive  | 41 (14.7)       | 8 (7.9)              | 33 (20.5)           |         |
| Unknown   | 16 (5.8)        | 12 (13.3)            | 30 (19.6)            |         |
| PI3KCA mut|                 |                       |                      |         |
| Negative  | 201 (72.3)      | 78 (86.7)             | 123 (80.4)           | 0.212   |
| Positive  | 42 (15.1)       | 12 (13.3)            | 30 (19.6)            |         |
| Unknown   | 35 (12.6)       | 12 (13.3)            | 30 (19.6)            |         |
| p110x     |                 |                       |                      |         |
| No overexpression | 186 (66.9)   | 64 (71.1)             | 122 (85.3)           | 0.119   |
| Overexpression | 40 (14.4)   | 19 (22.9)             | 21 (14.7)            |         |
| Unknown   | 52 (18.7)       | 25 (28.4)            | 35 (22.2)            |         |
| pAkt      |                 |                       |                      |         |
| No overexpression | 186 (66.9)   | 63 (71.6)             | 123 (77.8)           | 0.273   |
| Overexpression | 60 (21.6)   | 25 (28.4)             | 35 (22.2)            |         |
| Unknown   | 32 (11.5)       | 11 (14.4)            | 21 (14.7)            |         |
| p-mTOR    |                 |                       |                      |         |
| No overexpression | 161 (57.9)  | 58 (56.9)             | 103 (62.4)           | 0.367   |
| Overexpression | 106 (38.1) | 44 (43.1)             | 62 (37.6)            |         |
| Unknown   | 11 (4)          | 11 (11.1)            | 11 (11.1)            |         |
| pMAPK     |                 |                       |                      |         |
| No overexpression | 203 (73)   | 61 (74.6)             | 142 (92.8)           | <0.000  |
| Overexpression | 32 (11.5)  | 21 (25.6)             | 11 (7.2)             |         |
| Unknown   | 43 (15.5)       | 11 (11.1)            | 11 (11.1)            |         |
| p27       |                 |                       |                      |         |
| Loss      | 89 (32)         | 25 (26)               | 64 (39.5)            | 0.028   |
| Present   | 169 (60.8)      | 71 (74)               | 98 (60.5)            |         |
| Unknown   | 20 (7.2)        | 9 (10.0)              | 9 (10.0)             |         |

Abbreviations: EGFR = epidermal growth factor receptor; HR = hormone receptor; IGF1R = insulin-like growth factor 1-receptor; All comparisons by Chi^2 or Fisher tests.

Figure 2. Western blot results of Src and pSrc-Y416 (active) expression in BT474 parental and resistant cells. The expression of total Src did not change in resistant cells when compared with the parental cell line. In contrast, increased pSrc-Y416 was demonstrated in trastuzumab-resistant cells. Similar results were shown in SKBR3 cells (data not shown).

Table 4. Clinico-pathological, immunohistochemical and molecular features of trastuzumab untreated breast cancer patients (Kaplan–Meier; log-rank test)

| Variables | DFS   | P     | OS    | P     |
|-----------|-------|-------|-------|-------|
| Age <50 vs ≥50 years | 86% vs 73% | 0.061 | 89% vs 82% | 0.148 |
| Size <2 vs ≥2 cm | 85% vs 71% | 0.049 | 90% vs 79% | 0.095 |
| Grade (1+2 vs 3) | 84% vs 72% | NS   | 89.5% vs 80.6% | NS  |
| LVI (–) vs (+) | 76% vs 82% | NS   | 83% vs 91% | NS   |
| LN (–) vs (+) | 83% vs 69% | 0.021 | 88% vs 79% | 0.102 |
| Stage (I + II vs III) | 81% vs 61% | 0.004 | 88% vs 67% | 0.001 |
| CNS mets | 0% vs 0% | NS   | 40% vs 0% | NS   |
| Liver mets | 0% vs 0% | NS   | 33% vs 33% | NS   |
| Lung mets | 0% vs 0% | NS   | 50% vs 0% | 0.039 |
| ER (–) vs (+) | 67% vs 83% | 0.067 | 79% vs 87% | NS   |
| α-IGF1R | 76% vs 82% | NS   | 83% vs 89% | NS   |
| EGFR | 80% vs 57% | 0.003 | 87% vs 71% | 0.016 |
| PTEN loss | 72% vs 96% | NS   | 83% vs 100% | NS   |
| PI3KCA mutations | 77% vs 77% | NS   | 83.5% vs 90.5% | NS   |
| p110x | 100% vs 75% | NS   | 100% vs 83% | NS   |
| pAkt | 74% vs 79% | NS   | 74% vs 88% | NS   |
| p-mTOR | 73% vs 82% | NS   | 82% vs 89% | NS   |
| pMAPK | 78% vs 78% | NS   | 78% vs 86% | NS   |
| p27 | 75% vs 79% | NS   | 85% vs 82% | NS   |
| pSrc-Y416 | 76% vs 80% | NS   | 84% vs 85% | NS   |

Abbreviations: CNS = central nervous system, EGFR = epidermal growth factor receptor; ER = estrogen receptor; IGF1R = insulin-like growth factor 1-receptor; LN = lymph nodes; LVI = lymph vascular invasion; mets = metastasis.

nor with prognosis was found, in line with in vitro studies in trastuzumab-resistant and -sensitive cells (Liu et al, 2011). Therefore, this action appears to be independent of mTOR signalling. Other factors have also been recently associated with the mechanisms of trastuzumab resistance involving Src and PI3K signalling, such as EpoR/Jak2 (Liang et al, 2010), phosphorylation of beta1 integrin subunit upregulation and GDF15-mediated activation of TGF beta receptor-Src-HER2 signalling crosstalk (Joshi et al, 2011), but their clinical relevance has not been confirmed.
contrastumab treatment, especially in earlier stages. More correlated with shorter overall survival in patients under first line in metastatic disease vs vs Comparing our three groups of patients, adjuvant trastuzumab showed poorer outcome in patients under trastuzumab treatment. Furthermore, in our large cohort of patients, Src correlated with increased pSrc-Y416 levels in trastuzumab-resistant cells. In line, our experimental studies in BT474 and SKBR3 cell lines overall survival rates than patients having low active Src tumours. lower clinical response, a higher progressive disease and shorter series of 57 breast cancer patients treated with trastuzumab-based models that inhibition of Src enhanced trastuzumab-mediated has been involved in the mechanisms of resistance of et al et al. Recently, Zhang et al (2011) demonstrated in experimental models that inhibition of Src enhanced trastuzumab-mediated growth inhibition by promoting apoptosis. Further, in a small series of 57 breast cancer patients treated with trastuzumab-based therapies, high amounts of pSrc-Y416 in tumours correlated with lower clinical response, a higher progressive disease and shorter overall survival rates than patients having low active Src tumours. In line, our experimental studies in BT474 and SKBR3 cell lines showed increased pSrc-Y416 levels in trastuzumab-resistant cells. Furthermore, in our large cohort of patients, Src correlated with poorer outcome in patients under trastuzumab treatment. Comparing our three groups of patients, adjuvant trastuzumab vs first line in metastatic disease vs no trastuzumab, activated Src correlated with shorter overall survival in patients under trastuzumab treatment, especially in earlier stages. More interesting, however, was the fact that in subgroup analysis, those patients with HR-negative and increased pSrc-Y416 tumours had even more recurrences or died of the disease.

Preliminary preclinical as well as pharmacodynamic data suggest that Src inhibition is a viable therapeutic option in patients with Src-dependent neoplasms (Gnoni et al, 2011). Furthermore, it may represent a novel therapeutic strategy, with the potential to delay or prevent the acquisition of subsequent resistance to anti-growth factor therapies (Chen et al, 2011; Rexer et al, 2012). In summary, our data in acquired trastuzumab-resistant breast cancer cells and a large clinical series of patients with HER2 breast carcinoma reinforces that activation of Src in coexistence with alterations in the MAPK and PI3K/Akt pathways are associated with a lower response to trastuzumab. These findings argue in favour of Src as a potential therapeutic target in those patients. Moreover, considering that different resistance mechanisms may coexist in the same tumour, combination with other targeted agents with a potential for synergistic activity might be recommendable to restore sensitivity to trastuzumab. Nevertheless, further understanding of the mechanistic basis for progression of HER2-overexpressing breast cancer will allow more effective targeted treatment options to be developed.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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