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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.

In patients with HER2-positive breast carcinoma, the relationship between poor clinical response to trastuzumab and alterations of the PI3K/Akt pathway has been recently demonstrated. This pathway is activated by several growth factor receptors such as IGF1R, HER1 (EGFR) (Lu *et al*, 2001; Nahta *et al*, 2005), HER3 (Lipton *et al*, 2013), low PTEN levels or *PIK3CA*-activating mutations (Nagata *et al*, 2004; Lerma *et al*, 2008; Serra *et al*, 2011; Gallardo *et al*, 2012). Nevertheless, combinations of these alterations can justify the development of resistance only in a subset of tumours.

The *v-Src* (Rous sarcoma virus) tyrosine kinase was the first oncogenic gene discovered by Stehelin *et al* (1977). *c-Src* is the corresponding cellular gene that codifies Src, a non-receptor (intracellular) tyrosine kinase that belongs to the Src family kinases

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(Thomas and Brugge, 1997). The 60-kDa human c-Src tyrosine kinase contains two phosphorylation sites regulating its enzymatic activity. Phosphorylation at Tyr527 leads to a reduced activity, whereas autophosphorylation at Tyr416 is associated with full kinase activity (Roskoski, 2005). Src can be activated by extracellular signals such as integrins and growth factors, including PDGFR, HER family members (HER1; HER2 and HER3), IGF1R, hepatocyte growth factor/scatter factor receptor, mitogen-activated protein kinases – MAPK – and STAT3 pathways, leading to cellular survival and proliferation (Olayioye *et al*, 2001). Once activated, Src acts as a common upstream regulator of the Ras/MAPK (Mitra and Schlaepfer, 2006) and PI3K pathways (Ingley, 2008) inducing malignant transformation. In fact, activation of Src has been observed in about 50% of tumours from the colon, liver, lung, breast and pancreas (Dehm and Bonham, 2004).

Previous experimental results strongly indicate an important role of Src in the development and progression of breast cancer (Zou *et al*, 2009) as well as a viable therapeutic option (Gnoni *et al*, 2011). Moreover, novel studies performed in animal models, breast cancer cells (Lu *et al*, 2003; Wang *et al*, 2009; Boyer *et al*, 2012) and human tumours (Nagata *et al*, 2004; Liang *et al*, 2010; Zhang *et al*, 2011) have suggested Src as a key modulator of trastuzumab response and a common node downstream of multiple trastuzumab-resistance pathways. Although the available experimental data are in fact very promising, the results in clinical series have shortcomings due to the small number of patients included. Furthermore, the underlying molecular mechanisms of Src-mediated trastuzumab resistance remain unclear.

To address this issue, we analysed the Src activation (phosphorylated at Tyr416) status together with several other Src-dependent biomarkers and patients' outcome in a large series of HER2-positive breast carcinomas. Here we show that Src activation is associated with MAPK and PI3K/Akt oncogenic pathways and imply worse patients' outcome, indicating resistance. In addition, we confirmed the activity of phosphorylated-Src in two resistant HER2-positive breast cancer cell lines (SKBR3 and BT474).

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.

Clinical–pathological staging was performed according to the WHO system and tumours were graded according to the Elston and Ellis method. The most representative areas of the infiltrating tumour were selected for the tissue microarray (TMA) construction (Beecher Instruments; Silver Spring, MD, USA) with three 1-mm cores.

The follow-up of the trastuzumab-treated cohort was updated (Gallardo *et al*, 2012). Median patients' follow-up in group A was 8.4 years ($n=110$; range 0.26–17.9 years), in group B 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 o 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 TMAs 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 pharmaDx) 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. BT474 (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, Cölbe, 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% CO_2. Trastuzumab-resistant BT474 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 BT474 parental and trastuzumab-resistant cells were seeded in 96-well plates at a density of 1 or 2×10^3 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% CO_2. 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

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	PgR636	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 (Tyr416)	Rabbit Mn	1 : 50	Cell Sig	Citrate buffer pH 9	4 °C overnigth	Membr/cytopl, 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	Membr/cytopl, HS ⩾220
PTEN	6H2.1	1 : 50	Dako	Citrate buffer pH 6	20 min, RT	Membr/cytopl, HS ⩾75
p110α	Rabbit Pol	1 : 80	Cell Sig	Citrate buffer pH 9	4 °C overnigth	Membr/cytopl, HS ⩾150
pAkt (Ser473)	14-5	1 : 10	Dako	Citrate buffer pH 9	20 min, RT	Membr/cytopl, HS ⩾150
pmTOR (Ser2448)	Rabbit Pol	1 : 50	Cell Sig	Citrate buffer pH 9	20 min, RT	Membr/cytopl, HS ⩾30
pMAPK (Thr202/Tyr204)	20G11	1 : 100	Cell Sig	Citrate buffer pH 9	30 min, RT	Membr/cytopl, 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/cytopl = membrane/cytoplasm; Mn = monoclonal; Pol = polyclonal; PR = progesterone receptor. Dako, Dako Diagnostics (Glostrup, Denmark). NeoMarkers Inc (Fremont, CA, USA). Cell Signaling Technology (Danvers, MA, USA).

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% polyacrilamyde 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-Y416 #2101 (Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. After washing the membrane in PBS-Tween, it was incubated with secondary antibodies according to the primary antibody used, for 1 h at room temperature, washed three times in PBS-Tween and then visualised with enhanced chemiluminescence reagent, following the manufacturer's instructions (Amersham ECL Western Blotting Analysis System, GE Healthcare UK Limited, Little Chalfont, UK).

Statistical analyses. The chi-square or Fisher' tests were used to determine the distribution of the clinical–pathological, IHC and molecular characteristics. A receiver operating characteristic and area under the curve (AUC) were generated to determine a cutoff value of the protein expression and the potential clinical utility to predict trastuzumab response (recurrence *vs* death). Therefore, the cutoff with the largest AUC was chosen. Kaplan–Meier survival plots and log-rank tests were performed for the comparison of the survival curves. Multivariate analysis (Cox proportional hazards regression model) was carried out based on the results of the univariate analysis, to identify those factors that were independently associated with cancer-specific recurrence or death. Statistical analyses were performed with the SPSS/win 19.0 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, 33 (12%) were alive with disease, 85 (31%) died of the 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 (CNS; $P=0.009$). 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 α-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 α-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

Table 2. Clinico-pathological features in relation to pSrc-Y416

Variables	All cases, *n* (%)	pSrcY416 (+), *n* (%)	pSrcY416 (−), *n* (%)	*P-value
Number	278 (100)	105 (37.8)	173 (62.2)	
Age (years)				
<50	88 (31.7)	34 (36.2)	54 (32)	
⩾50	175 (62.9)	60 (63.8)	115 (68)	0.487
Unknown	15 (5.4)			
Size				
5–20 mm	103 (37.1)	45 (48.9)	58 (36.3)	
>20 mm	149 (53.6)	47 (51.1)	102 (63.8)	0.049
Unknown	26 (9.4)			
Grade				
1	12 (4.3)	2 (1.9)	10 (5.8)	
2	93 (33.5)	33 (31.4)	61 (35.3)	
3	171 (61.5)	70 (66.7)	102 (59)	0.203
Necrosis				
Absent	81 (29.1)	19 (35.8)	62 (52.5)	
Present	90 (32.4)	34 (64.2)	56 (47.5)	0.043
Unknown	107 (38.5)			
LVI				
Absent	203 (73)	70 (71.4)	133 (79.6)	
Present	62 (22.3)	28 (28.6)	34 (20.4)	0.127
Unknown	13 (4.7)			
LN status				
Negative	118 (42.4)	46 (46.9)	72 (43.4)	
Positive	146 (52.5)	52 (53.1)	94 (56.6)	0.573
Unknown	14 (5)			
Stage				
I	61 (21.9)	23 (22.8)	38 (22.6)	
II	109 (39.2)	39 (38.6)	70 (41.7)	
III	89 (32)	33 (32.7)	56 (33.3)	
IV	10 (3.6)	6 (5.9)	4 (2.4)	0.512
Unknown	9 (3.2)			

Abbreviations: LN = lymph nodes; LVI = lymph vascular invasion. *All comparisons by Chi^2 or Fisher tests.

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 focussed 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.

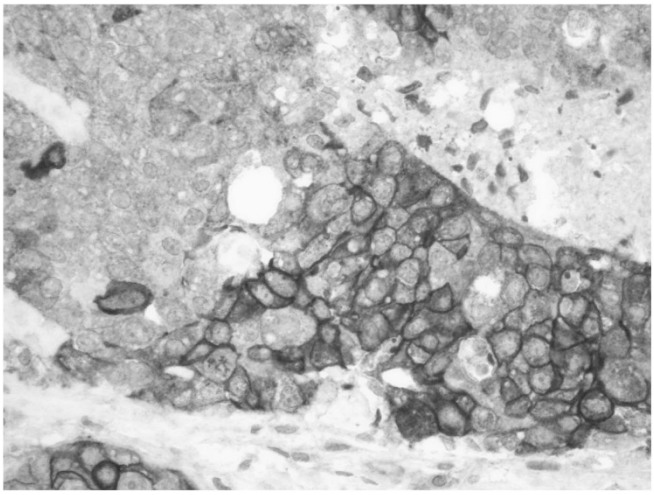

Figure 1. Representative example of the immunohistochemical expression of pSrc-Y416 (membrane ± cytoplasm) (× 400).

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 indicates 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*, 2012; Cizkova *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.

Despite the observed changes in mTOR levels in 39.7% of our cases, a significant association neither with Src activation status

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 Negative Unknown	150 (54) 118 (42.4) 10 (3.6)	51 (53.1 45 (46.9)	99 (57.6) 73 (42.4)	0.483
p53				
<20% ⩾20% Unknown	174 (62.6) 88 (31.7) 16 (5.8)	56 (56.6) 43 (43.4)	118 (72.4) 45 (27.6)	0.009
Ki67				
<14% ⩾14% Unknown	95 (34.2) 159 (57.2) 24 (8.6)	32 (33) 65 (67)	63 (40.1) 94 (59.9)	0.253
PTEN				
Loss Present Unknown	47 (16.9) 224 (80.6) 7 (2.5)	15 (14.9) 87 (85.3)	32 (18.9) 137 (81.1)	0.373
α-IGF1R				
No overexpression Overexpression Unknown	191 (68.7) 75 (27) 12 (4.3)	69 (67) 34 (33)	122 (74.8) 41 (25.2)	0.165
EGFR				
Negative Positive Unknown	221 (79.5) 41 (14.7) 16 (5.8)	93 (92.1) 8 (7.9)	128 (79.5) 33 (20.5)	0.006
***PIK3CA* mut**				
Negative Positive Unknown	201 (72.3) 42 (15.1) 35 (12.6)	78 (86.7) 12 (13.3)	123 (80.4) 30 (19.6)	0.212
p110α				
No overexpression Overexpression Unknown	186 (66.9) 40 (14.4) 52 (18.7)	64 (77.1) 19 (22.9)	122 (85.3) 21 (14.7)	0.119
pAkt				
No overexpression Overexpression Unknown	186 (66.9) 60 (21.6) 32 (11.5)	63 (71.6) 25 (28.4)	123 (77.8) 35 (22.2)	0.273
p-mTOR				
No overexpression Overexpression Unknown	161 (57.9) 106 (38.1) 11 (4)	58 (56.9) 44 (43.1)	103 (62.4) 62 (37.6)	0.367
pMAPK				
No overexpression Overexpression Unknown	203 (73) 32 (11.5) 43 (15.5)	61 (74.4) 21 (25.6)	142 (92.8) 11 (7.2)	<0.000
p27				
Loss Present Unknown	89 (32) 169 (60.8) 20 (7.2)	25 (26) 71 (74)	64 (39.5) 98 (60.5)	0.028

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

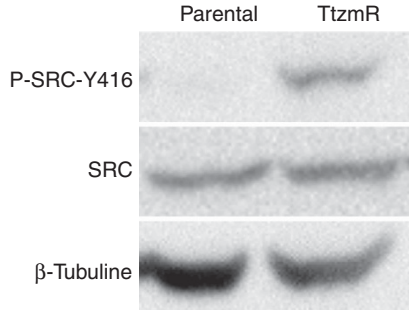

Figure 2. Western blot results of Src and pSrc-Y416 (active) expression in BT474 parental and resistant cells. The expression of total Src did not change in resistant cells when compared with the parental cell line. In contrast, increased pSrc-Y416 was demonstrated in trastuzumab-resistant cells. Similar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

	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
p110α	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* 79.5%	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 = oestrogen 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.

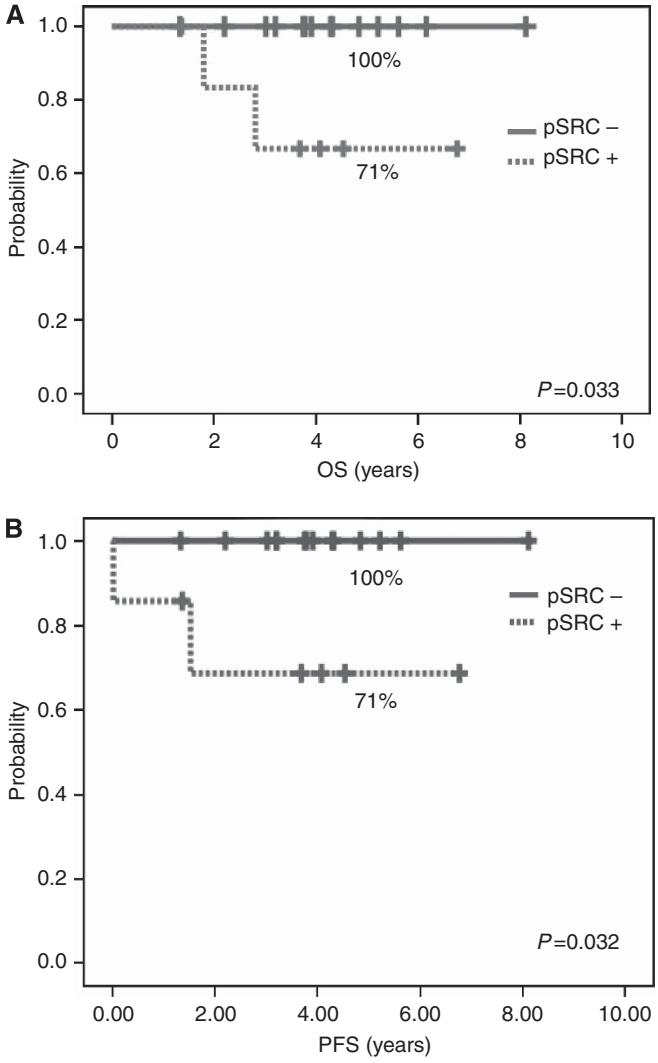

Figure 3. Kaplan-Meier plots for pSrc-Y416 results in patients under adjuvant trastuzumab therapy with HR-negative tumours: (**A**) overall survival, (**B**) disease-free survival.

p27, a key mediator of cell cycle arrest, is present in normal quiescent epithelial tissues. Loss of nuclear p27 was observed in 34.5% of our cases in correlation with HR-negative status, PTEN loss, high Ki67 and overexpression of p53 and EGFR but without impact or survival. Nevertheless, either p27 downregulation (Lane *et al*, 2001; Nahta *et al*, 2004) or upregulation (Huober *et al*, 2012) has been involved in the mechanisms of resistance of trastuzumab.

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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