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SHORT COMMUNICATION

# ERBB2 phosphorylation and trastuzumab sensitivity of breast cancer cell lines

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**Breast cancers that overexpress the ERBB2 tyrosine kinase receptor may be treated with the recombinant humanized monoclonal anti-ERBB2 antibody trastuzumab (herceptin). However, resistance to this targeted therapy is frequent. We have determined the response of 18 breast tumor cell lines to trastuzumab and compared it with the ERBB2 phosphorylation status using antibodies directed against tyrosine residue 1248. We show that sensitivity to trastuzumab is frequently associated with the expression of a phosphorylated ERBB2 protein.**
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A subset of breast cancers amplifies the *ERBB2* gene at chromosomal region 17q12 and overexpresses its protein product, the ERBB2 tyrosine kinase receptor (also called HER2). This alteration is associated with a poor disease outcome. The recombinant humanized monoclonal antibody trastuzumab (herceptin) is directed against the extracellular juxta-membrane region of ERBB2 (Cho *et al*., 2003). Patients with an ERBB2-amplified disease can benefit from trastuzumab treatment, alone or in combination with other drugs (Pegram and Slamon, 1999; Vogel *et al*., 2001; Baselga *et al*., 2004; Emens, 2005; Bernard-Marty *et al*., 2006; Gonzalez-Angulo *et al*., 2006). Randomized trials in ERBB2-positive early breast cancer have shown the clinical benefit of trastazumab (Marty *et al*., 2005; Mass *et al*., 2005; Piccart-Gebhart *et al*., 2005; Romond *et al*., 2005; Tan-Chiu *et al*., 2005). Unfortunately, not all ERBB2-positive breast cancers respond to this therapy. When administered as single agent and in a first-line setting against ERBB2-overexpressing or *ERBB2*-amplified tumors, the response rate is variable and clinical benefit (objective response and prolonged stable disease) ranges from one-third to half of the cases (Cobleigh *et al*., 1999; Vogel *et al*., 2002; Baselga *et al*., 2005).

The reasons for resistance to trastuzumab are not clear. It is probable that many factors modulate trastuzumab response *in vivo*. To study trastuzumab resistance, we have analysed the behavior of ERBB2-overexpressing breast cell lines (BCLs) cultured in the presence of trastuzumab. We determined their degree of sensitivity to trastuzumab and confronted it to the phosphorylation status of ERBB2.

## Selection and study of ERBB2-overexpressing BCLs

We assembled a panel of BCLs overexpressing the ERBB2 receptor and/or displaying *ERBB2* gene amplification. Selection was based on: (i) data from the literature (Kauraniemi *et al*., 2004; Lacroix and Leclercq, 2004; Orsetti *et al*., 2004); (ii) American Type Culture Collection database (http://www/atcc.org) and (iii) measurements of ERBB2 gene and protein status. Nineteen BCLs were analysed: BT-474, BT-483, HCC202, HCC1954, HCC2218, Hs578T, MCF7, MDA-MB-175, MDA-MB-361, MDA-MB-453, SK-BR-3, UACC-812, ZR-75–30 (http://www.atcc.org/), SUM-185, SUM-190, SUM-206, SUM-225 (Ethier *et al*., 1993, http://www.cancer.med.umich.edu/breast_cell/production), HME-1 (Clontech, Mountain View, CA, USA) and MCF7/ERBB2 (a gift from O Segatto, Rome). To obtain the latter cell line, the human ERBB2 cDNA cloned in pBabePuro was introduced in MCF7. The BCLs were grown using culture conditions recommended by their supplier.

*ERBB2* gene amplification was studied by fluorescence *in situ* hybridization (as described in Letessier *et al*., 2007) on metaphase spreads of BCL and by quantitative PCR amplification (Supplementary Table 1). *ERBB2* mRNA status was derived from Affymetrix microarray data (Charafe-Jauffret *et al*., 2006). ERBB2 protein expression was measured by immunohistochemistry (IHC) with the Dako (Glostrup, Denmark) Herceptest (http://www.dakousa.com).

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Not counting MCF7/ERBB2, 15 out of 18 BCL overexpressed ERBB2 mRNA and protein and 15 out of 18 BCLs showed increased gene copy number (Table 1).

## Trastuzumab response of BCLs

We treated 18 cell lines with increasing doses (0–100 $\mu$g/ml) of trastuzumab to determine their sensitivity to the drug. A dose–response analysis was done for each BCL (Supplementary Table 2). Representative dose–response curves are shown in Figure 1. Ten BCLs were sensitive and eight resistant. MCF7 and Hs578T, which do not overexpress ERBB2, were resistant, together with the MCF7/ERBB2 clone and five other cell lines (Table 1).

## ERBB2 phosphorylation status of BCLs

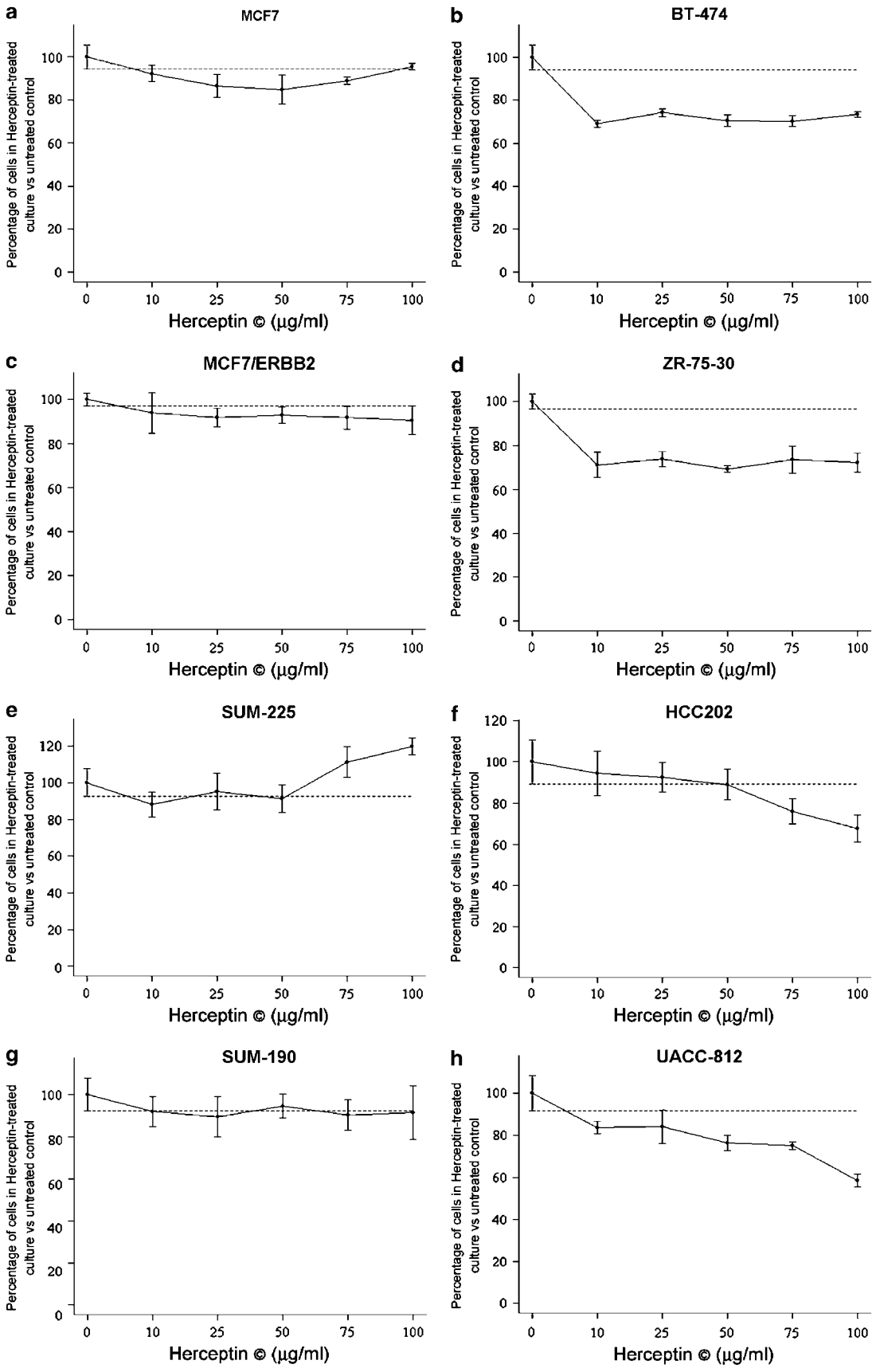
We determined the phosphorylation status of ERBB2 in BCL using an antibody directed against phosphotyrosine

**Table 1** ERBB2 status and sensitivity to trastuzumab of breast cell lines

| *Cell lines* | *Subtype*[a] | *T-T response*[b] | *ERBB2 gene status*[c] | *ERBB2 mRNA*[d] | *ERBB2 protein status*[e] | *ERBB2-phosphorylation status (WB)*[f] | *ERBB2-phosphorylation status (IHC)*[g] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | *Without T-T* | *After 6 h of T-T* | *After 72 h of T-T* |
| HME-1 | Basal* | ND | Not amp | NO | 0 | – | 0 | ND | ND |
| Hs578T | Basal* | Resistant | Not amp | NO | 0 | – | 0 | ND | ND |
| MCF7 | Luminal*/** | Resistant | Not amp | NO | 0 | – | 0 | 0 | 0 |
| MCF7/ERBB2 | ND | Resistant | ND | ND | + + | – | 0 | ND | ND |
| MDA-MB-453 | Luminal*/** | Resistant | AMP | Overexpressed | + + | – | 0 | 0 | 0 |
| SUM-185 | Luminal** | Resistant | AMP | Overexpressed | + + | – | 0 | 0 | 0 |
| SUM-190 | Basal** | Resistant | AMP | Overexpressed | + + + | P | + | + | + |
| SUM-206 | Basal* | Resistant | AMP | Overexpressed | + + + | – | + | ND | ND |
| SUM-225 | Basal*/** | Resistant | AMP | Overexpressed | + + + | P | + | + | + |
| BT-474 | Luminal*/** | Sensitive | AMP | Overexpressed | + + + | P | + + + | + | + |
| BT-483 | Luminal*/** | Sensitive | Not amp* | Overexpressed | + + + | – | + | ND | ND |
| CC1954 | Basal*/** | Sensitive | AMP | Overexpressed | + + + | P | + | + | + |
| HCC202 | Luminal** | Sensitive | AMP | Overexpressed | + + + | P** | ND | ND | ND |
| HCC2218 | ND | Sensitive | AMP | Overexpressed | + + + | P | + + + | ND | ND |
| MDA-MB-361 | Luminal*/** | Sensitive | AMP | Overexpressed | + + + | P | + + | ND | ND |
| MDA-MB-175 | Luminal*/** | Sensitive | Not amp | Overexpressed | + + | – | 0 | 0 | 0 |
| SK-BR-3 | Luminal*/** | Sensitive | AMP | Overexpressed | + + + | P | + + | + + | + |
| UACC-812 | Luminal*/** | Sensitive | AMP | Overexpressed | + + + | P | + + | + | + |
| ZR-75-30 | Luminal*/** | Sensitive | AMP | Overexpressed | + + + | P | + + + | + + | + |

Abbreviations: AMP, amplified; IHC, immunohistochemistry; ND, not done; NO, nonoverexpressed; Not amp, not amplified; P, phosphorylated-ERBB2/α-tubulin signal ratio from 0.14 to 1.55; T-T, trastuzumab treatment; WB, western blot; –: ratio from 0.005 to 0.03. [a]Molecular subtype was determined by using Affymetrix U133 Plus 2.0 human oligonucleotide microarrays (*Charafe-Jauffret *et al*., 2006 and unpublished; **Neve *et al*., 2006). [b]T-T. [c]*ERBB2* gene copy number/amplification was determined by quantitative PCR (Supplementary Table 1) and fluorescence *in situ* hybridization (FISH) analyses (data not shown). The two techniques showed concordant results. FISH was done with *HER2* FISH pharmDx kit (DakoCytomation, Glostrup, Denmark). Fluorescence was scored on 20 nonoverlapping nuclei per cell line to determine a ratio of *ERBB2*/cen-17, calculated by dividing the total number of red *ERBB2* signals by the total number of green cen-17 signals. Absence of amplification of BT-483 is taken from Neve *et al*. (2006). [d]*ERBB2* mRNA expression was determined as in a. [e]ERBB2 protein expression was determined by Dako-Herceptest and measured with the Dako scale (HercepTest kit scoring guidelines). [f]ERBB2 phosphorylation was determined by western blot. **Positivity was taken from Neve *et al*. (2006). [g]Herceptin was added to exponentially growing cells at a concentration of 50 $\mu$g/ml. Control cells were grown in drug-free medium. Cells were harvested by trypsinization after 6, 24 (data not shown), 48 (data not shown) and 72 h. Then, cells were washed with ice-cold phosphate-buffered saline buffer, counted with a cell counter (Beckman Coulter A[c]T diff analyser, Coulter Corporation, Miami, FL, USA) and used for preparation of cell pellet paraffin blocks. ERBB2-phosphorylation status determined by IHC was scored as follows: 0 (no expression), + (less than 10% of positive cells), + + (10% < positive cells < 50%), + + + (more than 50% of positive cells). Intensity was similar for all positive cells.

**Figure 1** Dose–response of breast tumor cell lines treated by trastuzumab. Sensitivity to trastuzumab was determined by dose–response analysis. Exponentially growing cells were harvested and plated on 96-well plates at 10 000 cells/well. After 24 h, herceptin (Genentech Inc., South San Francisco, CA, USA) was dissolved in sterile water at 20 mg/ml and added to the culture at concentrations of 10, 25, 50, 75 and 100 $\mu$g/ml. Fresh medium lacking herceptin was added to control wells of each cell line. After 72 h incubation, the medium was replaced with fresh drug-free medium. Suppression of cell growth was measured by using the CellTiter 96 AQueous One solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA). Viability of cells was calculated as the percentage of cells in trastuzumab-treated culture compared to untreated cells. All experiments were done in quadruplicates and mean $\pm$ s.d. of quadruplicates were calculated and plotted for each drug concentrations. A cell line was scored sensitive when mean−s.d. of quadruplicates of untreated cells was superior to mean + s.d. of quadruplicate of cells under treatment (dot line). (**a**, **c**, **e** and **g**) Examples of four resistant BCLs. (**b**, **d**, **f** and **h**) Examples of four sensitive BCLs.


a
MCF7
b
BT-474
c
MCF7/ERBB2
d
ZR-75-30
e
SUM-225
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HCC202
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SUM-190
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Percentage of cells in Herceptin-treated culture vs untreated control
Herceptin © (µg/ml)
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10
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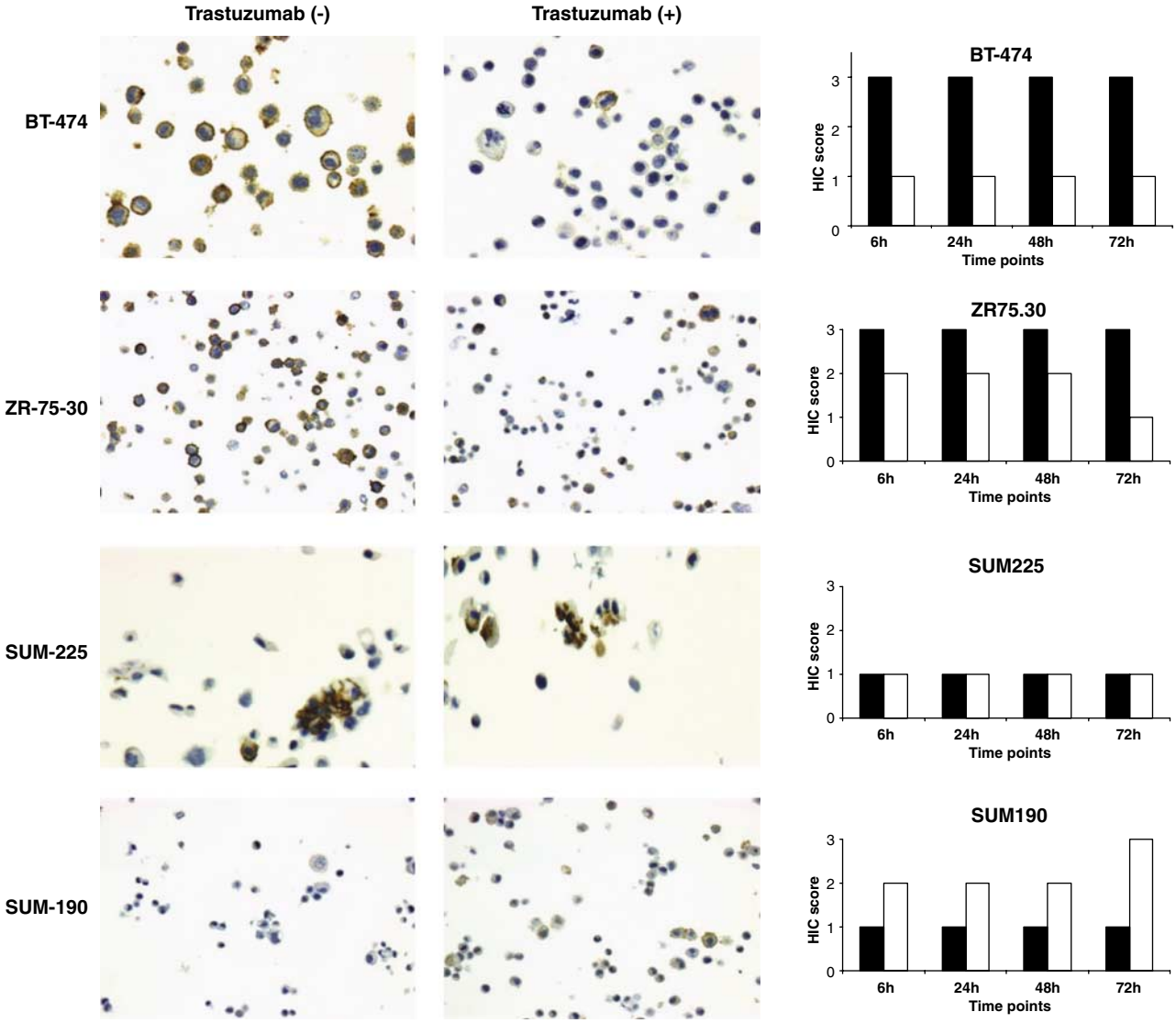


**Figure 2** ERBB2 phosphorylation status in breast cell lines treated or not by trastuzumab. (**a**) We determined by immunohistochemistry (IHC) the effect of trastuzumab on ERBB2 phosphorylation at various times. Cell pellet paraffin blocks were prepared as described previously (Charafe-Jauffret *et al*., 2006). Five-micrometer sections of cell pellet paraffin blocks were used for conventional IHC after transfer onto glass slides. Staining was done by using monoclonal mouse anti-human HER2-pY-1248 (clone PN2A, dilution: 1/20; DakoCytomation, Glostrup, Denmark) with CSA II biotin-free tyramide signal amplification system (DakoCytomation). Only membrane staining was considered and results were scored as described in Table 1. Overall, results show reduction of ERBB2 phosphorylation in sensitive BCLs and unchanged or increased ERBB2 phosphorylation in resistant BCLs. ERBB2 phosphorylation status before (first column) and after trastuzumab treatment (second column) of two sensitive BCLs (BT-474 and ZR-75-30) and two resistant BCLs (SUM-225 and SUM-190) are represented. To the right, IHC scores before and after trastuzumab treatment for each time point are represented on a histogram.

residue 1248 of the receptor. For most BCLs, we performed both IHC (Figure 2) and western blot (WB) (Figure 3) analyses. In the absence of trastuzumab treatment, 10- ERBB2-overexpressing cell lines, including eight sensitive and two resistant (SUM-190 and SUM-225), expressed a phosphorylated ERBB2 protein and five did not or expressed very low level (Table 1).

Finally, we determined the effect of herceptin treatment at various times of growth of 11 (five resistant and six sensitive) of the 19 BCLs (HME-1, SUM-206, MDA-MB-361, BT-483, Hs578T, HCC202, HCC2218 and MCF7/ERBB2 were not included). We measured ERBB2 phosphorylation after various times of treatment (Table 1, Figure 2 and Supplementary Figure 1). Overall, trastuzumab reduced ERBB2 phosphorylation in sensitive cell lines. For resistant cell lines, phosphorylation, when present, was low and remained unchanged. In the case of SUM-190, phosphorylation was more visible by IHC under treatment.

Thus, most (eight out of 10) cell lines sensitive to trastuzumab express a phosphorylated form of the ERBB2 receptor; this form can be affected by trastuzumab

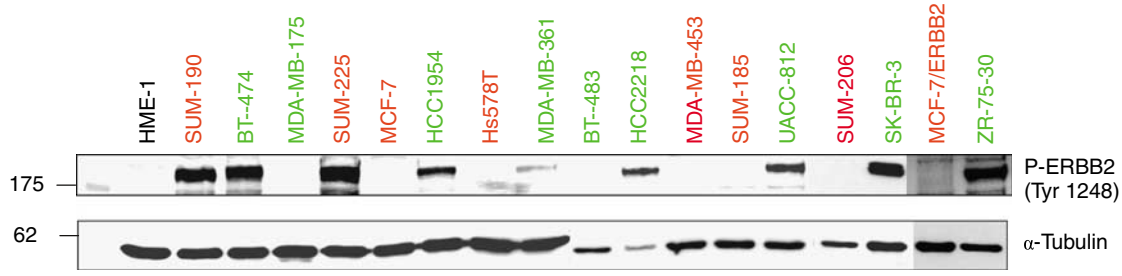


**Figure 3** Western blot analysis of ERBB2 phosphorylation in serum-starved breast cancer cell lines. Cells were washed with ice-cold phosphate-buffered saline after 18 h serum starvation (cell culture medium with 0.1% fetal bovine serum) and lysed in extraction buffer (1% (v/v) Triton X-100; 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7; 1 mM EDTA; 1 mM EGTA, 150 mM NaCl, 100 mM sodium fluoride, 1 mM Na3VO4 and one tablet of CompleteTM inhibitor mix (Roche Applied Science, Roche Diagnostics, Meylan, France) per 25 ml of buffer). Lysates were incubated on ice for 10 min and clarified by centrifugation (14 000 r.p.m. for 15 min at 4°C). Protein concentration was quantified by Protein Assay (Bio-Rad laboratories, Munich, Germany). A total of 100 $\mu$g of protein lysates were resolved by SDS–PAGE (7.5%) then transferred to nitrocellulose membrane. After blocking in TBS-T (saline buffer with 25 mM Tris–HCl containing 0.05% Tween-20) with 5% bovine serum albumin for 1 h at room temperature, blots were incubated sequentially with primary specific antibody (1:10 000) overnight at 4°C and secondary antibody anti-rabbit for 45 min at room temperature then detected by enhanced chemoluminescence–chemiluminescence (super signal WestPico; Pierce Biotechnology, Rockford, IL, USA). Antibodies used were anti-phospho-ERBB2 (Tyr1248; Upstate Biotechnology, Lake Placid, NY, USA) and anti-$\alpha$-tubulin (Sigma-Aldrich Chimie, Lyon, France) as control for variability of loading. Cell lines sensitive and resistant to trastuzumab are labeled in green and red, respectively. Molecular masses (kDa) of markers are shown in the left.

treatment. In contrast, four out of six resistant (MCF7/ERBB2, MDA-MB-453, SUM-185 and SUM-206) ERBB2-overexpressing BCLs did not express phosphorylated ERBB2 or expressed it weakly. These results suggest that ERBB2 phosphorylation in ERBB2-overexpressing cells is associated with more frequent drug efficiency. Trastuzumab resistance was seen not only with cells that do not overexpress ERBB2 (MCF7 and Hs578T) but also with cells that do not express phosphorylated ERBB2. However, two BCLs (SUM-190 and SUM-225) that express phosphorylated ERBB2 were resistant. Reciprocally, two BCLs (BT-483 and MDA-MB-175) that do not express phosphorylated ERBB2, or express low levels, were sensitive. Resistant cells with unphosphorylated overexpressing ERBB2 may have ceased to depend on ERBB2 signaling for growth and activated other downstream pathways. This is in agreement with the heterogeneous pattern of ERBB2 phosphorylation among tumor cells (data not shown).

## Further considerations

After hormone therapy, trastuzumab treatment of ERBB2-positive breast cancer is the second successful targeted therapy of the disease. To show the way for the use of other drugs, the mechanisms of trastuzumab activity and resistance must be understood. We can propose several explanations for the lack of strict correlation between sensitivity and phosphorylation. First, the anti-phospho-ERBB2 antibodies we used are probably not to detect the full spectrum of ERBB2 phosphorylation; sensitive BCLs may thus express forms of phosphorylated ERBB2 not detected here. There were also some differences in the assessment of phosphorylation status by IHC and WB. This was probably due to technical reasons and/or performance of the antibodies. Second, while the role of ERBB2 phosphorylation seems important, it is probably not the only factor involved in trastuzumab response. ERBB2-associated proteins may influence trastuzumab efficiency: receptors of the ERBB family (Diermeier *et al*., 2005), membrane receptors coregulated with ERBB2 such as CD44 (Wobus *et al*., 2001; Ghatak *et al*., 2005), CXCR4 (Li *et al*., 2004) and MUC4 (Ramsauer *et al*., 2003; Nagy *et al*., 2005), proteins of the ERBB2 signaling or endocytic pathways, or from cross-talking pathways (Nahta *et al*., 2006). Inactivation of PTEN, a cytoplasmic lipid-associated phosphatase involved in the AKT survival pathway, contributes to trastuzumab resistance (Nagata *et al*., 2004). Downregulation of p27/CDKN1B is associated with resistance (Nahta *et al*., 2004). Loss of RALT/ERRFI1 expression, a negative modulator of ERBB receptors, enhances trastuzumab resistance (Anastasi *et al*., 2005). Other regulators (Borg *et al*., 2000; Adélaïde *et al*., 2003; Badache *et al*., 2004) may also play a role. The *ERBB2*-amplified region includes several genes that may also participate in tumor progression under treatment (Bertucci *et al*., 2004). Genome analysis may reveal more factors associated with trastuzumab resistance (Neve *et al*., 2006). Third, ERBB2 structural alterations may play a role although only rare ERBB2 mutations have been found in breast carcinoma (Lee *et al*., 2006). Fourth, sensitivity was not strictly associated with any particular molecular subtype. In contrast to ERBB2-amplified tumors, which constitute a specific subtype, ERBB2-overexpressing cell lines are mostly distributed in basal and luminal subtypes (Charafe-Jauffret *et al*., 2006; Neve *et al*., 2006). However, basal cell lines were more frequently resistant.

In a previous study, a trend towards increased response was seen for patients with ERBB2-phosphorylated tumors (Hudelist *et al*., 2006). We observed a similar trend in a preliminary survey of trastuzumab-treated

cases. However, the relation between the clinical benefit of trastuzumab treatment and ERBB2 phosphorylation is difficult to establish in cohorts of patients who receive concomitant treatments. Even in conditions of monotherapy, the results may be difficult to interpret, as judged by our study of BCL. In the same previous report, the determination of the phosphorylation status of both ERBB2 and epidermal growth factor-receptor (EGFR) helped predict the response (Hudelist *et al*., 2006). Another study showed the efficiency of lapatinib (GW572016), a dual inhibitor of EGFR and ERBB2, to inhibit ERBB2 kinase in expressing cells was correlated with the level of ERBB2 expression and with the level of phosphorylated ERBB2 inhibition (Konecny *et al*., 2006). These and our results, and the fact that the IHC tests are easy to perform, should encourage systematic determination of ERBB2 phosphorylation status in the management of ERBB2-amplified breast cancers.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).