Correlation of Phosphorylated HER2 with Clinicopathological Characteristics and Efficacy of Trastuzumab Treatment for Breast Cancer

SNJEŽANA RAMIĆ[1], KSENIJA ASIĆ[1], MELITA PERIĆ BALJA[1], FRANE PAIĆ[2], VESNA BENKOVIĆ[3] and FABIJAN KNEŽEVIĆ[1]

[1]*Department of Pathology, University Hospital for Tumors, Sestre Milosrdnice Clinical Hospital Centre, Zagreb, Croatia;*
[2]*Laboratory for Epigenetic and Molecular Medicine, Department of Biology, School of Medicine, University of Zagreb, Zagreb, Croatia;*
[3]*Department of Animal Physiology, Division of Biology, Faculty of Science, University of Zagreb, Zagreb, Croatia*

Abstract. *Aim: To determine the correlation of phosphorylated human epidermal growth factor receptor-2 (pHER2) with clinicopathological characteristics of breast cancer (BC) and patients' response to trastuzumab-based therapy. Patients and Methods: pHER2 was determinated immuno-histochemically in 88 cases of HER2-positive and 50 cases of HER2-negative BC. All patients with HER2-positive BC received trastuzumab-based therapy and 16 of them (18.2%) had disease progression during therapy treatment (i.e. trastuzumab-resistant). Results: pHER2 was predominantly expressed in HER2-positive BC, with 55 cases (62.5%) of tumours expressing pHER2. Six cases of HER2-negative cancer (12.5%) displayed positive expression of pHER2. Expression of pHER2 correlated with younger age of patients and negative oestrogen receptor status. Acquisition of resistance to trastuzumab correlated with negativity for pHER2 (p=0.028). Conclusion: Positive expression of pHER2 may yield additional information regarding the poor prognosis of BC and could be used for pre-selection of patients with HER2-overexpressing BC displaying resistance to trastuzumab treatment.*

Human epidermal growth factor receptor-2 (HER2) is a transmembrane growth factor receptor belonging to the family of epidermal growth factor receptors (EGFR) that regulates cell growth and proliferation (1, 2). They are activated by binding of numerous ligands, leading to receptor dimerization and subsequent phosphorylation on the intracellular domain. Phosphorylation of receptors activates the downstream signalling pathways, resulting among others, in cell proliferation and migration (3, 4). Since HER2 is an orphan receptor (*i.e.* its ligand has not been identified), its activation takes place not just by ligand-mediated heterodimerization with other members of the EGFR family, but also by spontaneous ligand–independent homodimerization, especially in HER2-overexpressing tumours (5-7). Phosphorylation of HER2 (pHER2) is a precondition for downstream signalling and represents the functional and active form of HER2 (8). The main site of auto-phosphorylation of HER2 is on tyrosine 1248 residue (Tyr1248) (8, 9). Experiments on mammary tumours in HER2-positive transgenic mice showed that HER2 is usually present in the phosphorylated state (10). However, studies on HER2-positive breast cancer disagree on the percentage of HER2 in the phosphorylated state, reporting from 10% to 80% pHER2-positive cases (11-16). HER2 is overexpressed in approximately 20-25% of cases of invasive breast cancer (BC), resulting in tumours with more aggressive biological behaviour leading to poor prognosis, shorter survival and increased risk of death (1, 2). Patients with HER2-overexpressing tumours receive therapy with a monoclonal antibody against HER2, trastuzumab (Herceptin®; Roche). Binding of trastuzumab to the external domain of HER2 receptor leads to disruption of homodimerization and down-regulation of its signalling pathway (4, 17). In patients with HER2-overexpressing BC, trastuzumab-based therapy results in prolonged progression-free and overall survival (17, 18). However, although trastuzumab therapy is effective, still more than 30% of patients with HER2-positive tumours do not respond to this therapy (19). We studied the expression of active/phosphorylated HER2 in HER2-positive and -negative BC aiming to determine whether the receptor's activity (reflected by site-specific phosphorylation on Tyr1248) is

Correspondence to: Fabijan Knežević, MD, Ph.D., Pathologist, Department of Pathology, University Hospital for Tumors, Sestre Milosrdnice Clinical Hospital Centre, Ilica 197, 10000 Zagreb, Croatia. Tel: +38 513783588, e-mail: knezevicfabijan@yahoo.com

Key Words: Breast cancer, HER2, phosphorylation, trastuzumab.

potentially clinically useful, with respect to the efficacy of trastuzumab-based treatment and standard prognostic parameters for BC [size of the tumour, histological grade, oestrogen (ER) and progesterone (PR) receptor status and number of positive lymph nodes].

Patients and Methods

Patients. The study was performed on archived paraffin-embedded tissue samples obtained from 138 patients with primary ductal invasive BC, diagnosed and treated between 2008 and 2010 at the University Hospital for Tumours, Zagreb, Croatia. According to HER2 status, available from pathological reports, 88 patients were diagnosed with positive and 50 patients with negative HER2 status. Standard clinicopathological parameters (tumour size, histological grade, lymph node status, ER and PR status) and the age of patients were retrieved from the pathological reports. ER and PR were expressed as a percentage of stained cells (Table I). The cut-off value for hormone receptors was 10%. All HER2 positive cases included in the study received trastuzumab-based therapy for at least one year. First-line therapy was chemotherapy combined with trastuzumab (according to the treatment protocols) and then trastuzumab as single agent in 3-week cycles up to 17 cycles or more. Patients were selected for trastuzumab-based therapy according to immunohistochemical (IHC) HER2 3+ score (77 cases) or gene amplification confirmed by chromogenic *in situ* hybridization (CISH) in HER2 2+ cases (11 cases). Resistance to trastuzumab recorded as metastatic progression of the disease during trastuzumab therapy, which was detected using radiological methods as distant metastasis to liver, bones or brain, was taken from hospital charts. Sixteen out of 88 (18.2%) patients who received trastuzumab-based therapy patients had progression of disease.

Immunohistochemical method. Immunohistochemical staining was performed on archived, formalin-fixed and paraffin-embedded tumour tissues (donor block), prepared as tissue micro array blocks (Tissue-Tek®Quick Ray™ System; Sakura, Japan). Using a core needle, three 2-mm size cores of chosen areas were taken from each donor block and placed into a recipient block. After one hour at 60˚C, prepared blocks with multiple tissue samples were embedded in paraffin. Tumour tissue microarrays were cut in 2-3 μm sections, deparaffinized in xylene, and rehydrated through alcohol to distilled water. After drying, sections were then heated in a water-bath for 20 min at 97˚C in a target retrieval solution, pH 9.0 (S2367; Dako, Glostrup, Denmark). Prior to immunohistochemical staining, sections were treated with peroxidase blocking solution for 5 min. After rinsing with buffer, the sections were incubated with the primary mouse monoclonal antibody against phosphorylated HER2 on Tyr1248 (HER2-pY-1248, clone PN2A; Dako) at 1:40 dilution overnight at 4˚C, followed by immunohistochemical staining with universal secondary antibody conjugated with horseradish peroxidase (EnVision Flex/HRP High pH; Dako). Antibody PN2A is highly specific for the activated tyrosine-phosphorylated form of HER2 (pY-Tyr1248) and does not cross-react with closely related receptors (15). Subsequently, sections were incubated with 3,3'-diaminobenzidine (DAB) chromogen, counterstained with hematoxylin, dehydrated, cleared, and cover-slipped. Paraffin-embedded recombinant human EGFR stimulated SKBR-3 BC cell line was used as positive control (Dako). Negative controls were obtained by omitting the primary antibody. Positive reaction of PN2A primary antibody is membranous, with possible cytoplasmic staining. Expression of pHER2 was assessed using semi-quantitative scoring method of the HercepTest™ (0=no staining or weak membranous staining in fewer than 10% of tumour cells, 1+=weak, fragmented membranous staining, 2+=moderate and 3+=strong membranous staining in more than 10% of tumour cells). Tumours that expressed moderate (2+) to strong (3+) membranous staining pattern in more than 10% of tumour cells were considered as pHER2 expressors. Tumours that expressed weak membranous staining (1+) or only cytoplasmic staining were considered negative. Expression of pHER2 was scored in three cores from each tumour and the average score was used.

Table I. *Clinicopathological features of 138 patients with human epidermal growth factor receptor-2-overexpressing (HER2-positive) and HER2-non-expressing (HER2-negative) primary breast cancer.*

Clinicopathological feature	HER2-positive n=88	HER2-negative n=50
Age (years)		
Mean (±Std. dev.)	55.7 (±11.2)	60.0 (±13.6)
Tumour size (mm)		
Mean (±Std. dev.)	26.4 (±17.3)	29.3 (±15.1)
Histological grade (n, %)		
II	38 (43.2)	29 (58)
III	50 (56.8)	17 (34)
Positive lymph node status (n, %)	54 (61.4)	25 (50)
Hormone receptor status (n, %)*		
ER positive	46 (52.3)	44 (88)
PR positive	35 (39.8)	32 (64)
Response to trastuzumab therapy (n, %)		
Sensitive	72 (81.8)	-
Resistant	16 (18.2)	-

*ER: Oestrogen receptor; PR: progesterone receptor.

Statistical analysis. To determine significant associations between the expression of pHER2 and clinicopathological parameters, Student's *t*-test for quantitative variables (age and tumour size) and Chi square test with Yates correction for qualitative variables grouped as positive or negative (ER and PR, status of lymph nodes and trastuzumab resistance) were used. Spearman's analysis was used to correlate the pHER2 and response to trastuzumab with prognostic parameters within the HER2-positive group. Analyses were performed using StatSoft software Statistica 7.0 (Tulsa, OK, USA) with the level of statistical significance set at $p<0.05$.

Results

Out of 138 patient tumour tissues samples included in the study, pHER2 was positive in 61 tumours (44.2%) with strong membranous staining in 33 samples and moderate staining in 28 samples. pHER2 was negative in 77 tumours (55.8%), with weak staining in 43 samples and without staining in 34 tissue samples. Figure 1 shows pHER2

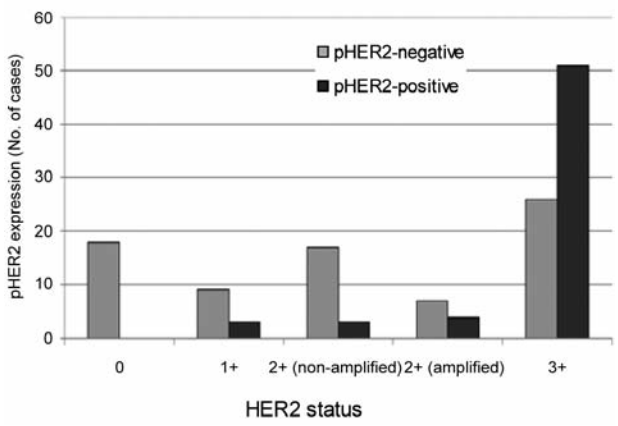

Figure 1. *Distribution of phosphorylated human epidermal growth factor receptor 2 (pHER2) expression in relation to HER2 score according to the Hercep Test and gene amplification status. pHER2-positive tumours were predominantly score 3+ in 51/77 (66.3%) of cases and score 2+ with gene amplification (amplified) in 4/11 (36.4%). pHER2-negative tumours were score 0 in 18 cases (100%), score 1+ in 9/12 (36.4%) and 2+ without gene amplification (non-amplified) in 17/20 (85%).*

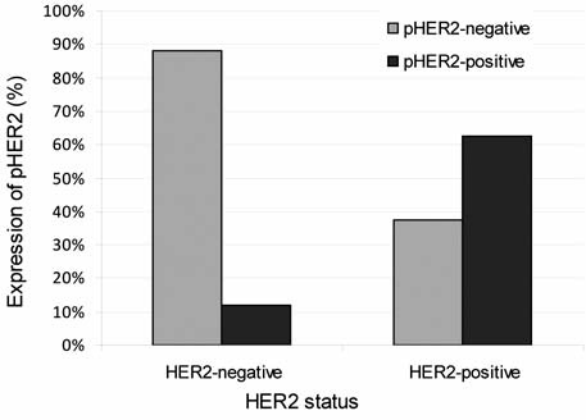

Figure 2. *Percentage of phosphorylated human epidermal growth factor receptor-2 (pHER2)-positive tumours in relation to total HER2 status. pHER2 was positive in 62.5% of HER2-positive tumours and in 12% of HER2-negative tumours (χ^2=30.95, p<0.001).*

expression according to HER2 score. pHER2 was positive in 62.5% (55/88) of HER2-positive tumours, with strong membranous staining in 31 and moderate staining in 24. pHER2 was negative in 33 tumours, with weak staining in 30 tumours and without staining in three. pHER2 was predominantly positive in HER2-positive tumours compared to HER2-negative tumours (χ^2=30.95, p<0.001) (Figure 2). In the HER2-negative group, 88% of tumours displayed a non-phosphorylated form of HER2 (Figure 2). pHER2 was positive in only six tumours (12%), with strong membranous staining in two and moderate staining in four. Negative pHER2 status was found in 44 tumours, with weak staining in 13 tumours and without staining in 31.

Association of pHER2 with clinicopathological parameters. Table II presents differences in clinicopathological characteristics between pHER2-positive and -negative tumours. Patients with pHER2-positive tumours were younger than those with pHER2-negative tumours (p=0.006). Although not statistically significant, pHER2-positive tumours were smaller in size than pHER2-negative ones (p=0.061). pHER2-positive tumours were less differentiated (grade III in 66% of cases *vs*. 46.7% grade III in pHER2-negative tumours), but grade and lymph node status did not differ between pHER2-positive and -negative tumours. The expression of pHER2 inversely correlated with positivity for ER: positive ER was found in 49.2% of pHER2-positive tumours *vs*. 77.9% of ER-positive tumours with negative pHER2 (p<0.001).

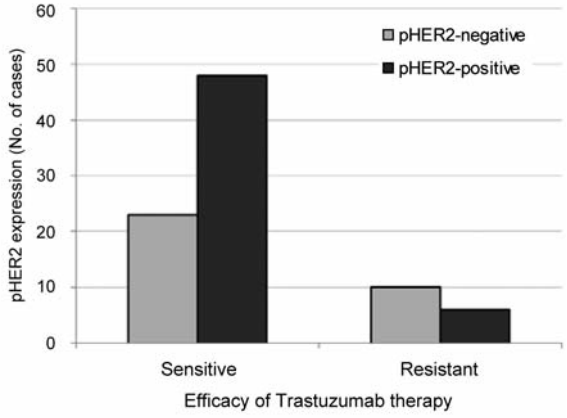

Figure 3. *Number of phosphorylated human epidermal growth factor receptor-2 (pHER2) positive tumours in relation to efficacy of trastuzumab-based therapy. pHER2 was positive in 49/72 (68.1%) of trastuzumab-sensitive tumours while only 6/16 (37.5%) of trastuzumab-resistant tumours showed pHER2 positivity (χ^2=4.84, p=0.028).*

Association of pHER2 expression with trastuzumab resistance. Acquisition of resistance to trastuzumab correlated with negativity for pHER2 (p=0.028). Trastuzumab-resistant tumours expressed lower levels of pHER2, with only 37.5% (6/16) of cases having positive pHER2 compared to 68.1% (49/72) of pHER2-positive cases in trastuzumab-sensitive tumours (Figure 3). Only four trastuzumab-sensitive tumours (5.5%) had complete absence of immunohistochemical staining. The mean size of trastuzumab-resistant tumours was 34.8 mm compared to 24.7 mm for trastuzumab-sensitive tumours (t-test, p=0.032). Spearman correlation analysis revealed positive correlation of resistance with the number of

Table II. *Differences in clinicopathological features between phosphorylated human epidermal growth factor receptor-2-positive (pHER2-positive) and pHER2-negative primary breast cancer.*

Clinicopathological feature	pHER2-positive n=61	pHER2-negative n=77	*p*-Value
Age (years)			
Mean (±Std. dev.)	54.0 (±10.5)	59.7 (±13.0)	0.006*
Tumour size (mm)			
Mean (±Std. dev.)	24.5 (±12.3)	29.8 (±19.1)	0.061*
Histological grade (n, %)			
II	29 (34.0)	38 (49.4)	(χ^2=0.03)
III	31 (66.0)	36 (46.7)	0.862†
Lymph node status (n, %)			
Positive	33 (54.1)	46 (59.7)	(χ^2=0.24)
Negative	28 (45.9)	31 (40.3)	0.623†
Oestrogen receptor status (n, %)			
Positive	30 (49.2)	60 (77.9)	(χ^2=11.16)
Negative	31 (50.8)	17 (22.1)	<0.001†
Progesterone receptor status (n, %)			
Positive	26 (42.6)	41 (53.2)	(χ^2=1.14)
Negative	35 (57.4)	36 (46.8)	0.285†

**t*-test; †χ^2 test with Yates correction.

positive lymph nodes (R=0.292, p=0.006), tumour size (R=0.224, p=0.038) and although not statistically significant, with tumour grade (R=0.179, p=0.099). Thirteen patients (81.3%) who did not benefit from trastuzumab therapy had positive lymph nodes at the time of diagnosis. Resistant tumours had the lowest level of ER positivity, with only 31.3% (5/16) of cases being ER-positive. Acquisition of resistance correlated with ER-negative status, but this was not statistically significant (R=0.190, p=0.079).

Discussion

HER2 is overexpressed in 20-25% of BC cases (1). In pathological conditions, its ability to form dimers with all receptors from the EGFR family results in uncontrolled growth and migration of tumour and poor prognosis (2, 3). Ligand-independent homodimerization is a preferred mechanism in HER2-overexpressing tumours and a reason for receptor deregulated phosphorylation/activation and oncogenic transformation of cells (4-6). Experiments *in vitro* on HER2-positive BC cells and on mammary tumours in HER2-positive transgenic mice showed that HER2 is always found in the phosphorylated state (13, 21, 22). Our work showed that pHER2 was predominantly expressed in HER2-positive tumours, with 62.5% positive cases, indicating that when HER2 is overexpressed, it is commonly activated (p<0.001). Our results are similar to those reported in studies by Cicenas *et al*. (8) and Hayashi *et al*. (23), where pHER2 expression was found in 68-73% of HER2-positive tumours. Yet results from other studies reported discordant percentages of pHER2 positivity in HER2-positive BC (9, 13, 16). In studies by DiGiovanna *et al*. (2, 14) and Thor *et al*. (15), pHER2 positivity was observed in only 10-12% of the HER2-positive cases. A discrepancy between their and our results may be due to differences in the case cohorts, modifications in immunohistochemical methodology or different cut-off values used for evaluation of pHER2 positivity. However, it still remains to be answered why HER2 may be overexpressed but not activated. Many studies reported that HER2-overexpressing tumours "prefer" homodimerization and Tyr1248 phosphorylation of HER2 is the main autophosphorylation site (11, 21). The finding of 37.5% tumours negative for pHER2 among HER2-positive tumours in our study could be explained by possible receptor mutations in the phosphorylation domain or by phosphorylation on other sites through heterodimerization with other receptors of the EGFR family rather than homodimerization (21). Interestingly, not all tumours that were pHER2-positive had a HER2 overexpression. In fact, we found that in six cases (12%) of HER2-negative tumours, pHER2 was positive, and 13 tumours (26%) even had weak pHER2 expression. This weak pHER2 expression correlated with weak-to-moderate expression of HER2 on the cell membrane, which was negative according to the Hercep test score (Figure 1). Our results confirmed findings by Cicenas *et al*. (8) where 27% of HER2-negative cases expressed pHER2. Similar findings were reported by Wulfkuhle *et al*. (24), who found pHER2-positive/fluorescence *in situ* hybridization-negative tumours

using quantitative protein microarray assays. These results support the hypothesis that even lower levels of HER2 may be sufficient to elicit a potent mitogenic signal (25, 26). The finding of low HER2-expressing tumours with positive pHER2 could also indicate HER2 activation through heterodimerization, which is able to activate HER2 in cases of low expression. This was confirmed in a study by Frogne *et al*. (27), where 83% of HER2-negative cases with pHER2-positive expression also had positive expression of HER1 and HER3. Moreover, expression of pHER2 in low HER2-expressing tumours indicated that these tumours had HER2-mediated growth, and thus may be potential responders to anti-HER2 therapy. In BC cell lines, Menendez *et al*. showed that trastuzumab can be effective in low HER2-expressing tumours (25). Our findings differed from a study of Thor *et al*. (15), who failed to detect pHER2 in 509 BC cases without HER2 overexpression. Multiple studies have shown that pHER2 is a predictor of poor response and survival in BC (2, 8, 15, 23, 27, 28). In a study of Thor *et al*. (15), HER2-positive cases with positive pHER2 had lower survival rates than the HER2-positive but pHER2-negative ones. They suggested that pHER2 expression could be used for a selection of patients with BC with a more aggressive course of disease and shorter survival. Expression of pHER2 with HER2 expression could allow for more accurate prognosis. Since survival analysis was not the subject of this research, we cannot firmly confirm this theory. We did not find any significant difference of tumour grade or number of positive lymph nodes between pHER2-positive and -negative tumours. pHER2-positive tumours were mostly of grade III (66% *vs*. 46.7%), but with fewer positive lymph nodes (54.1% *vs*. 59.7%) than pHER2-negative. Positive pHER2 strongly correlated with ER-negativity ($p<0.001$) and with younger age of patients ($p=0.006$). Interestingly, some authors reported correlation of pHER2 with younger age, higher grade, negativity for ER or negativity for PR (8, 15, 27), while some failed to report any correlation of pHER2 with clinicopathological characteristics of BC (29). Contrary to previous results, we found a negative correlation of pHER2 with tumour size (15). This discrepancy suggests that pHER2 positivity could be a marker for tumours with higher potency for growth and migration. This statement is supported by the theory of DiGiovanna *et al*. (28), who reported that HER2 is very active in early tumourigenesis, suggesting that such tumours have aggressive biological behaviour from the outset. Heterogeneity between pHER2 and HER2 expression presented in Figure 1, demonstrates that positive pHER2 expression cannot be used as a surrogate for HER2 positivity. HER2 overexpression is mandatory for trastuzumab therapy, but it is not sufficient alone for efficacy. Although trastuzumab represents the most widely used anti-HER2 therapy, many patients with HER2-overexpression do not benefit from this therapy (16, 19). Recent studies have shown that trastuzumab inhibits HER2 homodimerization, but does not reduce HER2 phosphorylation (21, 30). One possible explanation may be that in trastuzumab-resistant tumours, HER2 signalling through homodimerization is no longer a main pathway. There are several currently proposed molecular mechanisms for trastuzumab resistance and some of them are dependent on pHER2 formation (truncated, but constitutively active p95HER2, HER2 mutations leading to kinase-active receptor, cross-activation by other growth factor receptors) (4, 17, 18, 19). In our study, 16 out of 88 patients who received trastuzumab therapy (18.2%) had distant metastases. Furthermore, pHER2 expression significantly differed between trastuzumab-resistant and -sensitive cases. Trastuzumab-resistant tumours were more frequently pHER2-negative, with 62.5% pHER2-negative cases *vs*. 31.9% pHER2-negative cases in trastuzumab-sensitive tumours ($p=0.028$). The predictive value of pHER2 in BC was also reported by Giuliani *et al*., where 89% of pHER2-positive cases had obtained a response to trastuzumab therapy *vs*. 49% of pHER2-negative ones (31). Moreover, previous studies demonstrated that progression-free survival in trastuzumab-based treatment more than doubled in patients with pHER2-positive tumours, compared with patients lacking pHER2 positivity (20, 22, 29, 31). In our study, trastuzumab-resistant tumours were larger then trastuzumab-sensitive ones, with more positive lymph node involvement. We found that trastuzumab-resistant tumours had lower expression of ER than did trastuzumab-sensitive tumours. Only Ross *et al*. (20) reported that ER-positive tumours have a better response to trastuzumab treatment. Crosstalk between ER and HER2 signalling pathways is also one proposed mechanisms of trastuzumab resistance (18, 19). Overall, expression of activated HER2 gives additional information on the biological behaviour of HER2-overexpressing tumours. Heterogeneity in pHER2 and HER2 expression demonstrates that pHER2 cannot be used as a surrogate for HER2. Still, expression of pHER2 could be used for pre-selection of patients for trastuzumab-based therapy. Combined with HER2 positivity, pHER2-positive cases could be categorized as good candidates for trastuzumab therapy. Patients with HER2-positive/pHER2-negative tumours could be categorized as poor candidates, who would not respond to trastuzumab therapy and perhaps who could benefit from double anti-tyrosine kinase therapy in first-line treatment (lapatinib, pertuzumab). The question remains whether patients with low HER2 expression but pHER2-positive tumours are candidates for any therapy with tyrosine kinase inhibitors. Our study confirms that pHER2 is a marker of poor prognosis and characterizes tumours of higher malignant potential.

References

1 Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, Dowsett M, Fitzgibbons PL, Hanna WM, Langer A, McShane LM, Paik S, Pegram MD, Perez EA, Press MF, Rhodes A, Sturgeon C, Taube SE, Tubbs R, Vance GH, van de Vijver M, Wheeler TM and Hayes DF; American Society of Clinical Oncology: College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol *25*: 118-125, 2007.

2 DiGiovanna MP, Stern DF, Edgerton SM, Whalen SG, Moore II D and Thor AD: Relationship of epidermal growth factor receptor expression to ERBB-2 signalling activity and prognosis in breast cancer patients. J Clin Oncol *23*: 1152-1160, 2005.

3 Wolf-Yadlin A, Kumar N, Zhang Y, Hautaniemi S, Zaman M, Kim H-D, Grantcharova V, Lauffenburger DA, and White FM: Effects of HER2 overexpression on cell signalling networks governing proliferation and migration. Mol Syst Biol *2*: 54, 2006.

4 Nahta R: Molecular mechanisms of trastuzumab-based treatment in HER2-overexpressing breast cancer. ISRN Oncology *2012*: 428062, 2012.

5 Desmedt C, Sperinde J, Piette F, Huang W, Jin X, Tan Y, Durbecq V, Larsimont D, Giuliani R, Chappey C, Buyse M, Winslow J, Piccart M, Sotiriou C, Petropoulos C and Bates M: Quantitation of HER2 expression or HER2:HER2 dimers and differential survival in a cohort of metastatic breast cancer patients carefully selected for trastuzumab treatment primarily by FISH. Diagn Mol Pathol *18(1):* 22-29, 2009.

6 Shi Y, Huang W, Tan Y, Jin X, Dua R, Penuel E, Mukherjee A, Sperinde J, Pannu H, Chenna A, DeFazio-Eli L, Pidaparthi S, Badal Y, Wallweber G, Chen L, Williams S, Tahir H, Larson J, Goodman L, Whitcomb J, Petropoulos C and Winslow J: A novel proximity assay for the detection of proteins and protein complexes: Quantitation of HER1 and HER2 total protein expression and homodimerization in formalin-fixed, paraffin-embedded cell lines and breast cancer tissue. Diagn Mol Pathol *18(1)*: 11-21, 2009.

7 Spears M, Taylor KJ, Munro AF, Cunningham CA, Mallon EA, Twelves CJ, Cameron DA, John JT and Bartlett MS: In situ detection of HER2:HER2 and HER2:HER3 protein-protein interactions demonstrate prognostic significance in early breast cancer. Breast Cancer Res Treat *132(2)*: 463-470, 2012.

8 Cicenas J, Urban P, Küng W, Vuaroqueaux V, Labuhn M, Wight E, Eppenberger U, Eppenberger-Castori S: Phosphorylation of tyrosine 1248-ERBB2 measured by chemiluminescence-linked immunoassay is an independent predictor of poor prognosis in primary breast cancer patients. Eur J Cancer *42*: 636-645, 2006.

9 Taniyama K, Ishida K, Toda X, Motoshita J, Kuraoka K, Saito A, Tni Y, Uike T, Teramoto S and Koseki M: Tyrosine 1248-phosphorylated HER2 expression and HER2 gene amplification in female invasive ductal carcinomas. Breast Cancer *15(3)*: 231-240, 2008.

10 DiGiovanna MP, Lerman MA, Coffey RJ, Muller WJ, Cardiff RD and Stern DF: Active signaling by Neu in transgenic mice. Oncogene *17*: 1877-1884, 1998.

11 Bates M, Sperinde J, Leitzel K, Koestler W, Fuchs E, Ali S, Weidler J, Wu Y, DeFazio-Eli L, Frankson K, Winslow J, Chappey C, Huang W and Lipton A: Quantitative HER2 homodimer levels correlate with time to first recurrence in HER2-positive breast cancer patients who did not receive trastuzumab in the adjuvant setting. Cancer Res *69(2 Suppl)*: SABCS-1074, 2009.

12 Bates M, Sperinde J, Köstler WJ, Ali SM, Leitzel K, Fuchs EM, Paquet A, Lie Y, Sherwood T, Horvat R, Singer CF, Winslow J, Weidler JM, Huang W and Lipton A: Identification of a subpopulation of metastatic breast cancer patients with very high HER2 expression levels and possible resistance to trastuzumab. Ann Oncol *22*: 2014-2020, 2011.

13 DeFazio-Eli L, Strommen K, Dao-Pick T, Parry G, Goodman L and Winslow J: Quantitative assays for the measurement of HER1-HER2 heterodimerization and phosphorylation in cell lines and breast tumors: Applications for diagnostics and targeted drug mechanism of action. Breast Cancer Res *13*: R44, 2011.

14 DiGiovanna MP, Carter D, Flynn SD and Stern DF: Functional assay for HER-2/*neu* demonstrate active signaling in a minority of HER-2/neu-overexpressing invasive human breast tumours. Br J Cancer *74*: 802-806, 1996.

15 Thor AD, Liuv S, Edgerton S, Moore D 2nd, Kasowitz KM, Benz CC, Stern DF and DiGiovanna MP: Activation (tyrosine phosphorylation) of ERBB-2 (HER-2/*neu*): A study of incidence and correlation with outcome in breast cancer. J Clin Oncol *18*: 3230-3239, 2000.

16 Hudelist G, Köstler WJ, Czerwenka K, Kubista E, Attems J, Müller R, Gschwantler-Kaulich D, Manavi M, Huber I, Hoschützky H, Zielinski CC, Singer CF: HER2/neu and EGFR tyrosine kinase activation predict the efficacy of trastuzumab-based therapy in patients with metastatic breast cancer. Int J Cancer *118*: 1126-1134, 2006.

17 Hudis CA: Trastuzumab – mechanism of action and use in clinical practice. N Eng J Med *357*: 39-51, 2007.

18 Dean-Colomb W and Esteva FJ: HER2-positive breast cancer: Herceptin and beyond. Eur J Cancer 44(18): 2806-2812, 2008.

19 Fiszman GL and Jasnis MA: Molecular mechanisms of trastuzumab resistance in HER2-overexpressing breast cancer. Int J Breast Cancer *2011*: 352182, 2011.

20 Ross JS, Slodkowska EA, Symmans WF, Puszal L, Ravdin PM and Hortobagyi GN: The HER-2 receptor and breast cancer: Ten years of targeted anti-HER2 therapy and personalized medicine. Oncologist *14*: 320-368, 2009.

21 Ghosh R, Narasanna A, Wang SE, Liu S, Chakrabarty A, Balko JM, González-Angulo AM, Mills GB, Penuel E, Winslow J, Sperinde J, Dua R, Pidaparthi S, Mukherjee A, Leitzel K, Kostler WJ, Lipton A, Bates M and Arteaga CL: Trastuzumab has preferential activity against breast cancers driven by HER2 homodimers. Cancer Res *71(5)*: 1871-1882, 2011.

22 Ginestier C, Adélaïde J, Gonçalvès A, Repellini L, Sircoulomb F, Letessier A, Finetti P, Geneix J, Charafe-Jauffret E, Bertucci F, Jacquemier J, Viens P and Birnbaum D. ERBB2 phosphorylation and trastuzumab sensitivity of breast cancer cell lines. Oncogene *26*: 7163-7169, 2007.

23 Hayashi N, Iwamoto T, Gonzalez-Angulo AM, Ferrer-Lozano J, Lluch A, Niikura N, Bartholomeusz C, Nakamura S, Hortobagyi GN and Ueno NT: Prognostic impact of phosphorylated HER-2 in HER-2+ primary breast cancer. Oncologist *16(7)*: 956-965, 2011.

24 Wulfkuhle J, Pierobon M, Laird J, Espina V, Liotta L, Esserman L, Petricoin E: Discovery of a new phospho-HER2+/FISH– molecular subtype of human breast cancer by functional pathway mapping. J Clin Oncol *27*: 15s, 2009.

25 Menendez JA, Mehmi I and Lupu R: Trastuzumab in combination with heregulin-activated HER-2/neu (ERBB2) triggers a receptor-enhanced chemosensitivity effect in the absence of HER2/neu overexpression. J Clin Oncol *24*: 3735-3746, 2006.

26 Arteaga CL: Can trastuzumab be effective against tumors with low HER2/Neu (ERBB2) receptors? J Clin Oncol *24(23)*: 3722-3725, 2006.

27 Frogne C, Laenkholm AV, Lyng MB, Henriksen KL and Lykkesfeldt AE: Determination of HER2 phosphorylation at tyrosine 1221/1222 improves prediction of poor survival for breast cancer patients with hormone receptor-positive tumors. Breast Cancer Res *11*: 1186-1200, 2009.

28 DiGiovanna MP, Chu P, Davison TL, Davidson TL, Howe CL, Carter D, Claus EB and Stern D: Active signaling by HER-2 in a subpopulation of HER-2-overexpressing ductal carcinoma *in situ*: Clinicopathological correlates. Cancer Res *62*: 6667-6673, 2002.

29 Hudelist G, Köstler WJ, Attems J, Czerwenka K, Müller R, Manavi M, Steger GG, Kubista E, Zielinski CC, Singer CF: HER-2/*neu*-triggered intracellular tyrosine kinase activation: *in vivo* relevance of ligand-independent activation mechanisms and impact upon the efficacy of trastuzumab-based treatment. Br J Cancer *89*: 983-991, 2003.

30 Gijsen M, King P, Perera T, Parker PJ, Harris AL, Larijani B and Kong A: HER2 phosphorylation is maintained by a PKB negative feedback loop in response to anti-HER2 Herceptin in breast cancer. PLoS Biol *8(12)*: e1000563, 2010.

31 Giuliani R, Durbecq V, Di Leo A, Paesmans M, Larsimont D, Leroy JY, Borms M, Vindevoghel A, Jerusalem G, D'Hondt V, Dirix L, Canon JL, Richard V, Cocquyt V, Majois F, Reginster M, Demol J, Kains JP, Delree P, Keppens C, Sotiriou C, Piccart MJ and Cardoso F: Phosphorylated HER-2 tyrosine kinase and HER-2/*neu* gene amplification as predictive factors of response to trastuzumab in patients with HER-2-overexpressing metastatic breast cancer. Eur J Cancer *43*: 725-735, 2007.

Received March 8, 2013
Revised April 17, 2013
Accepted April 22, 2013