Non-phosphorylated Tyr-1248 form of human epidermal growth factor receptor 2 (HER2) predicts resistance to trastuzumab therapy and poor disease-free survival of HER2-positive breast cancer patients

Ramić, Snježana; Paić, Frane; Smajlbegović, Velda; Perić Balja, Melita; Hiršl, Lea; Marton, Ingrid; Knežević, Fabijan

Source / Izvornik: **Croatian Medical Journal, 2022, 63, 126 - 140**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

https://doi.org/10.3325/cmj.2022.63.126

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:105:519799

RESEARCH ARTICLE

CMJ

Croat Med J. 2022;63:126-40
https://doi.org/10.3325/cmj.2022.63.126

Non-phosphorylated Tyr-1248 form of human epidermal growth factor receptor 2 (HER2) predicts resistance to trastuzumab therapy and poor disease-free survival of HER2-positive breast cancer patients

Snježana Ramić[1], Frane Paić[2], Velda Smajlbegović[3], Melita Perić Balja[1], Lea Hiršl[4], Ingrid Marton[5], Fabijan Knežević[5]

[1]Department of Oncological Pathology, University Hospital for Tumors, Sestre Milosrdnice University Hospital Center, Zagreb, Croatia

[2]Laboratory for Epigenetic and Molecular Medicine, Department of Medical Biology, University of Zagreb School of Medicine, Zagreb, Croatia

[3]Oncology Clinic, Clinical Center of Sarajevo University, Sarajevo, Bosnia and Herzegovina

[4]Center for Proteomics, Faculty of Medicine, University of Rijeka, Rijeka, Croatia

[5]Department of Pathology, Sveti Duh University Hospital, Zagreb, Croatia

Aim To determine the predictive value of phosphorylated human epidermal growth factor receptor 2 (pHER2^{Y1248}) status in breast cancer (BC) patients undergoing trastuzumab-based adjuvant therapy.

Methods Immunohistochemical status of pHER2^{Y1248}, EGFR/HER1, HER3, and HER4 was determined in 124 consecutive HER2-positive BC patients (median age [range] = 57 years [49.0-64.0]) treated at the University Hospital for Tumors, Zagreb, between 2008 and 2011. The median follow-up was 84 months (60.0-84.0). Prognostic factors of disease free survival (DFS) rate were evaluated with Kaplan-Meier/log-rank test and Cox regression analysis.

Results pHER2^{Y1248}, HER1, HER3, and HER4 were expressed in 66.1%, 9.7%, 70.2%, and 71.0% of patients, respectively. Disease progression (DP) was observed in 17.1% of pHER2^{Y1248}-positive and 47.6% of pHER2^{Y1248}-negative BCs ($P = 0.001$). Kaplan-Meier analysis showed a worse five-year DFS in pHER2^{Y1248}-negative patients who were older than 60 years ($P < 0.001$) and had positive lymph node status ($P < 0.001$); tumor size >2.0 cm ($P < 0.001$); higher histological grade ($P < 0.001$); HER2E intrinsic subtype ($P < 0.001$), negative hormone receptors ($P < 0.001$); negative HER1 status ($P < 0.001$), positive HER3 ($P = 0.002$); and/or positive HER4 ($P = 0.002$) status. The only negative prognostic factor for five-year DFS in multivariate Cox regression analysis was pHER2^{Y1248}-negative (hazard ratio [HR] 3.6, 95% confidence interval [CI] 1.8-7.2, $P < 0.001$) and lymph node-positive status (HR 3.6, 95% CI 1.3-9.8, $P = 0.014$).

Conclusion pHER2^{Y1248} predicts sensitivity to trastuzumab and a better five-year DFS regardless of any other prognostic parameter. In HER2-positive BC patients. Non-phosphorylated HER2^{Y1248} is a strong predictor of trastuzumab resistance and a poor DFS.

Received: April 1, 2021

Accepted: September 27, 2021

Correspondence to:
Frane Paić
Laboratory for Epigenetics and Molecular Medicine
Department of Biology and Medical Genetics
School of Medicine, University of Zagreb
Šalata 3
10 000 Zagreb, Croatia
fpaic@mef.hr

Until the development of trastuzumab, a highly-specific monoclonal antibody targeted against human epidermal growth factor receptor 2 (HER2), breast cancer (BC) with positive HER2 was an aggressive and rapidly proliferating malignancy with a poor prognosis. HER2 is overexpressed in about 20% of BC patients, and trastuzumab reduces the risk of disease recurrence almost by half (1-4). However, a subset of HER2-positive BC patients fails to benefit from such therapy (1-6). Resistance to trastuzumab-based therapy was recorded in almost 30% of patients, and different resistance mechanisms have been described (1-7). Besides, HER2-positive BC is very heterogeneous and includes tumors with positive (luminal type) and those with negative (HER2-enriched type) estrogen- (ER) and progesterone- (PgR) hormone receptor status (5). Thus, the approach to BC treatment is not uniform, and the equal response to therapy or the same mechanisms of resistance cannot be expected (4-7). HER2 is a member of the epidermal growth factor receptor (ErbB) family of four human receptor tyrosine kinases (ErbB1/EGFR/HER1, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4). Ligand binding on the extracellular receptor domain leads to its homo- or hetero-dimerization, resulting in its activation/phosphorylation on the receptors' cytoplasmic domain (4,6,8,9). Although without a known ligand, HER2 is the preferred and the most potent dimerization partner due to its high catalytic activity. Active HER2 has several phosphorylation sites. In cases of overexpression (due to gene amplification as in HER2-positive BC), tyrosine-1248 (pHER2^{Y1248}) is the most potent site because it is constitutively activated as a consequence of HER2 homodimerization. Other phosphorylation sites are usually activated by heterodimerization (6,8,9). So far, HER2 status is the only validated biomarker for anti-HER2 therapy in BC patients (1-3). However, detection of gene amplification or protein overexpression may not truly reflect the activated status of HER2. We assumed that the phosphorylation status of HER2 was a true indicator of its activity and that its heterodimerization with other ErbB family members might contribute to trastuzumab resistance. Therefore, the study aimed to evaluate the predictive value of pHER2^{Y1248} coexpressed with other ErbB family members and hormone receptors in HER2-overexpressing BC patients postoperatively treated with trastuzumab-based therapy.

PATIENTS AND METHODS

Patients

This retrospective study was performed on treatment-naive, archived formalin-fixed paraffin-embedded (FFPE) tumor tissues surgically removed from 124 consecutive patients diagnosed with HER2-positive primary ductal invasive breast cancer (BC). The patients were treated at the University Hospital for Tumors, Zagreb, between 2008 and 2011. All patients received adjuvant trastuzumab-based therapy for at least one year. Demographic and clinicopathological data were retrieved from medical records. Disease-free survival (DFS) rate was defined as the time in months from the date of surgery to the date of disease progression (DP). Data on disease progression, revealed by radiological methods (ultrasound, magnetic resonance, or positron emission tomography–computed tomography) as local recurrence or distant metastases, were obtained from the clinical database. Patients with other complications during trastuzumab therapy were not included. The follow-up period was over 84 months with the last check-up performed in May 2018. All participants signed the informed consent. The study was approved by the Ethics Committee of the University Hospital for Tumors (EP-15506/11-6).

Immunohistochemical staining

pHER2^{Y1248} and ErbB family members were immunohistochemicaly analyzed on FFPE samples prepared as tissue microarray blocks (Tissue-Tek®Quick Ray System; Sakura, Japan). All BC samples are routinely processed immediately after surgery to avoid the potential loss of epitopes due to a delayed time to fixation. FFPE blocks were processed as previously described (10). Shortly, 3-µm thick serial microarray tissue sections were heated in a water bath for 20 min at 97 °C in an antigen retrieval solution, pH 9.0 (S2367; Dako, Glostrup, Denmark). The sections were incubated (overnight, 4 °C) with primary antibodies (pHER2^{Y1248}: clone PN2A [Dako, Glostrup, Denmark] dilution 1:25; EGFR/HER1: clone E30 [Dako], dilution 1:50; HER3: clone DAK-H3-IC [Dako], dilution 1:50; HER4: clone sc-283 [Santa Cruz Biotechnology, Inc., Texas, USA], dilution 1:40). Subsequently, the sections were incubated (45 minutes, room temperature [RT]) with secondary antibody conjugated with horseradish peroxidase (EnVision Flex/HRP High pH; Dako) followed by incubation (10 minutes, RT) in DAB chromogen (3,3'-diaminobenzidine; Dako) and counterstained with hematoxylin.

The expression of pHER2^{Y1248} was assessed according to the semiquantitative HercepTest scoring method (11,12). BCs expressing moderate to strong membranous staining in more than 10% of cells were considered pHER2^{Y1248}-positive (10). Tumors without staining, with weak membranous staining, or with only cytoplasmic staining were considered pHER2^{Y1248}-negative.

The HercepTest method was also applied for immunohistochemical analysis of EGFR/HER1, HER3, and HER4 with BCs exhibiting 2+ or 3+ membranous/cytoplasmic staining considered as positive.

Statistical analysis

The normality of distribution was assessed with the Shapiro-Wilk test. Data are presented as frequencies or median and interquartile range (IQR). Relationships between immunohistochemical data and clinicopathological parameters were assessed with the Spearman correlation (r), *t* test, and χ^2, or Fisher exact test. The Kaplan-Meier/log-rank test was used to assess the difference in five-year DFS rate between the patient subgroups. Univariate and multivariate Cox proportional hazards model was used to determine the independent prognostic effect of individual variables on DFS rate, with the results presented as hazard ratio (HR) and 95% confidence interval (CI). All statistical tests were two-sided. The intergroup differences with $\alpha<0.05$ were considered significant and corrected according to the Bonferroni procedure (the corrected level of significance is Pc = 0.05/N; N- number of independent tests). Statistical analysis was performed with SPSS, trial version (IBM Corp., Armonk, NY, USA).

TABLE 1. Demographic and clinical characteristics of HER2-positive breast cancer (BC) patients before adjuvant trastuzumab-based therapy*

Characteristic	N (%)
Follow-up period; months, median (IQR)	84 (60.0-84.0)
Age of patients; years, median (IQR)	57 (49.0-64.0)
Age (years)	
<60	77 (62.1)
≥60	47 (37.9)
Tumor size; cm, median (IQR)	2.1 (1.6-3.0)
Tumor size stratification (cm)	
<2.0	50 (40.3)
≥2.0	74 (59.7)
Histological grade	
II	54 (43.5)
III	70 (56.4)
Estrogen receptor	
positive	67 (54.0)
negative	57 (46.0)
Progesterone receptor	
positive	50 (40.3)
negative	74 (59.7)
Intrinsic subtypes	
luminal B	70 (56.5)
HER2E	54 (43.5)
Lymph node status	
positive	75 (60.5)
negative	49 (39.5)
Disease progression	
present	34 (27.4)
absent	90 (72.6)

*Abbreviations: HER2 – human epidermal growth factor receptor 2; luminal B – estrogen receptor and/or progesterone receptor-positive and HER2 positive; HER2E – estrogen receptor and/or progesterone receptor-negative and HER2-positive intrinsic subtype; IQR – interquartile range.

RESULTS

Patients' characteristics

The median age at the time of surgery was 57 years. The majority of patients were younger than 60 years (62.1%). Tumors >2.0 cm were present in 59.7% of patients. BC samples were mostly classified as histological grade III (56.45%) and were associated with positive axillary lymph nodes (60.5%) at the time of surgery (Table 1).

The median follow-up was 84 months, and 34/124 (27.4%) patients had disease progression (DP). Among them, the median time to DP was 23.5 months (IQR 18.0-34.5). Positive expression of ER and PgR was detected in 54% and 40.3% of patients, respectively (Table 1). Thus, patients were classified in two intrinsic subtypes: luminal B (ER- and/or PgR-positive) and HER2E (ER- and PgR-negative), with 56.5% of patients belonging to the luminal B subtype (Table 1).

Immunohistochemical expression of pHER2 and ErbB family members

The pHER2^{Y1248}-positive staining was detected in 82 patients (2+ and 3+ staining status was 29.8% and 36.3%, respectively, Table 2). Although 42 (33.9%) patients were considered pHER2^{Y1248}-negative, a total absence of staining was observed in only 9 (7.2%) patients.

The rates for HER1- and HER3-positive staining were 9.7 and 70.2, respectively (Table 2). Among HER1-positive patients, 4 showed moderate and 8 strong membranous immunoreactivity. Among HER3-positive patients, 54 (43.6%) showed moderate and 33 (26.6%) showed strong membranous staining. Predominantly cytoplasmic HER4-positive staining was identified in 88 (71.0%) patients (29.8% moderate and 41.2% strong positive staining), while 29.0% were negative (Figures 1 and 2).

Association of expression of pHER2 and ErbB family members with clinicopathological parameters

The pHER2^{Y1248} status did not significantly correlate with tumor size (r = 0.037; *P* = 0.683), lymph node status (r = -0.056, *P* = 0.539), histological grade (r = -0.010, *P* = 0.912), intrinsic subtype (r = -0.113, *P* = 0.211), or the expression of ER (r = -0.147, *P* = 0.103) and PgR (r = -0.037, *P* = 0.683) (Table 2 and Table 3).

TABLE 2. Association of pHER2 and other epidermal growth factors (ErbB) family members with clinicopathological prognostic features of breast cancer (BC) patients. The values are presented as frequencies and percentages*

	pHER2			ErbB1/HER1			ErbB3/HER3			ErbB4/HER4		
Variable	negative N = 42 (33.9)	positive N = 82 (66.1)	*P*†	negative N = 112 (90.3)	positive N = 12 (9.7)	*P*†	negative N = 37 (29.8)	positive N = 87 (70.2)	*P*†	negative N = 36 (29.0)	positive N = 88 (71.0)	*P*†
Age of patients (years)												
<60	19 (45.2)	58 (70.7)	0.007	70 (62.5)	7 (58.3)	1.000	19 (51.4)	58 (66.7)	0.156	20 (55.6)	57 (64.8)	0.415
≥60	23 (54.8)	24 (29.3)		42 (37.5)	5 (41,7)		18 (48.6)	29 (33.3)		16 (44.4)	31 (35.2)	
Size of tumor (mm)												
<20	18 (42.9)	32 (39.0)	0.703	46 (41.1)	4 (33.3)	0.761	16 (43.2)	34 (39.1)	0.693	16 (44.4)	34 (38.6)	0.687
≥20	24 (57.1)	50 (61.0)		66 (58.9)	8 (66.7)		21 (56.8)	53 (60.9)		20 (55.6)	54 (61.4)	
Histological grade												
II	18 (42.9)	36 (43.9)	1.000	48 (42,9)	6 (50.0)	0.762	13 (35.1)	41 (47.1)	0.241	13 (36.1)	41 (46.4)	0.323
III	24 (57.1)	46 (56.1)		64 (57.1)	6 (50.0)		24 (64.9)	46 (52.9)		23 (63.9)	47 (53.4)	
Intrinsic subtype												
HER2E	15 (35.7)	39 (47.6)	0.252	47 (42.0)	7 (58.3)	0.362	15 (40.5)	39 (44.8)	0.696	19 (52.8)	35 (39.8)	0.232
lum B	27 (64.3)	43 (52.4)		65 (58.0)	5 (41.7)		22 (59.5)	48 (55.2)		17 (47.2)	53 (60.2)	
Lymph node status												
negative	15 (35.7)	34 (41.5)	0.566	43 (38.4)	6 (50.0)	0.538	12 (32.4)	37 (42.5)	0.322	15 (41.7)	34 (38.6)	0.840
positive	27 (64.3)	48 (58.5)		69 (61.6)	6 (50.0)		25 (67.6)	50 (57.5)		21 (58.3)	54 (61.4)	
Disease progression												
negative	22 (52.4)	68 (82.9)	0.001‡	80 (71.4)	10 (83.3)	0.509	26 (70.3)	64 (73.6)	0.826	29 (80.6)	61 (69.3)	0.269
positive	20 (47.6)	14 (17.1)		32 (28.6)	2 (16.7)		11 (27.9)	23 (26.4)		7 (19.4)	27 (30.7)	
pHER2^{Y1248}												
negative				41 (36.6)	1 (8.3)	0.058	16 (43.2)	26 (29.9)	0.213	9 (25.0)	33 (37.5)	0.214
positive				71 (63.4)	11 (91.7)		21 (56.8)	61 (70.1)		27 (75.0)	55 (62.5)	
ErbB1/HER1												
negative	41 (97.6)	71 (86.6)	0.058				33 (89.3)	79 (90.8)	0.750	32 (88.9)	80 (90.9)	0.744
positive	1 (2.4)	11 (13.4)					4 (10.8)	8 (9.2)		4 (11.1)	8 (9.1)	
ErbB3/HER3												
negative	16 (38.1)	21 (25.6)	0.213	33 (29.5)	4 (33.3)	0.750				11 (30.6)	26 (29.5)	1.000
positive	26 (61.9)	61 (74.4)		79 (70.5	8 (66.7)					25 (69.4)	62 (70.5)	
ErbB4/HER4												
negative	9 (21.4)	27 (32.9)	0.214	32 (28.6)	4 (33.7)	0.744	11 (29.7)	25 (28.7)	1.000			
positive	33 (78.6)	53 (67.1)		80 (71.4)	8 (66.3)		26 (70.3)	62 (71.3)				
ER												
negative	15 (35.7)	42 (51.2)	0.128	50 (44.6)	7 (58.3)	0.544	15 (40.5)	42 (48.3)	0.440	21 (58.3)	36 (40.9)	0.112
positive	27 (64.3)	40 (48.8)		62 (55.4)	5 (41.7)		22 (59.5)	45 (51.7)		15 (41.7)	52 (59.1)	
PgR												
negative	24 (57.1)	50 (61.0)	0.703	65 (58.0)	9 (75.0)	0.358	24 (64.9)	50 (57.5)	0.549	24 (66.7)	50 (56.-8)	0.323
positive	18 (42.9)	32 (39.0)		47 (42.0)	3 (25.0)		13 (35.1)	37 (42.5)		12 (33.3)	38 (43.2)	

*Abbreviations: HER – human epidermal growth factor receptor; lum B –luminal B (estrogen receptor (ER) and/or progesterone receptor (PgR)-positive and HER2 positive); HER2E – ER- and PgR-negative and HER2-positive intrinsic subtype; pHER2^{Y1248} – tyrosine 1248-phosphorylated HER2.
†Bonferroni non-adjusted *P* values.
‡Significant *P* values (<Pc). Bonferroni correction for multiple comparisons Pc = 0.004 (0.05/12 - number of comparisons). Pearson chi square or Fisher exact test were used as appropriate.

pHER2^{Y1248}-positive patients were more frequently HER3-positive, HER4-positive, hormone receptor-negative, and HER1-negative (Table 2). Thus, pHER2^{Y1248}-positive patients were more commonly classified as luminal B (52.4%) than as HER2E (47.6%) (Table 2). pHER2^{Y1248} status significantly negatively and weakly correlated with younger age (r = -0.25, $P = 0.005$) and DP (r = -0.32; $P < 0.001$). However, after Bonferroni correction only the relationship with DP remained significant (Table 2). There were 70.7% of women younger than 60 years among pHER2^{y1248}-positive patients and 45.2% among pHER2^{y1248}-negative patients. DP was observed in only 17.1% of pHER2^{Y1248}-positive and in 47.6% of pHER2^{Y1248}-negative BCs (Table 2).

Among the standard clinicopathological characteristics, DP positively and weakly correlated with positive lymph node status (r = 0.31; $P < 0.001$) and higher histological grade (r = 0.25; $P = 0.005$) (Table 4). However, after the Bonferroni correction the relationship remained significant only for positive lymph node status (Table 4).

TABLE 3. Association of pHER2 and hormone receptors (ER and PgR) family members with clinicopathological prognostic features of breast cancer (BC) patients. The values are presented as frequencies and percentages*

	ER			PgR		
Variable	negative N = 57 (46.0)	positive N = 67 (54.0)	P†	negative N = 74 (59.7)	positive N = 50 (40.3)	P†
Age of patients (years)						
<60	37 (44.9)	40 (59.7)	0.582	43 (58.1)	34 (68.0)	0.346
≥60	20 (35.1)	27 (40.3)		31 (41.9)	16 (32.0)	
Size of tumor (cm)						
<2.0	18 (31.6)	32 (47.8)	0.098	25 (33.8)	25 (50.0)	0.093
≥2.0	39 (68.4)	35 (52.2)		49 (66.2)	25 (50.0)	
Histological grade						
II	21 (36.8)	33 (49.3)	0.204	29 (39.2)	25 (50.0)	0.270
III	36 (63.2)	34 (50.7)		45 (60.8)	25 (50.0)	
Intrinsic subtype						
HER2E	54 (94.7)	0 (0,0)	-	54 (73.0)	0 (0.0)	-
luminal B	3 (5.3)	67 (100.0)		20 (27.0)	50 (100.0)	
Lymph node status						
negative	24 (42.1)	25 (37.3)	0.713	29 (39.2)	20 (40.0)	1.000
positive	33 57.9)	42 (62.7)		45 (60.8)	30 (60.0)	
Disease progression						
negative	38 (66.7)	52 (77.6)	0.226	51 (68.9)	39 (78.0)	0.309
positive	19 (33.3)	15 (22.4)		23 (31.1)	11(22.0)	
pHER2Y1248						
negative	15 (26.3)	27 (40.3)	0.128	24 (32.4)	18 (36.0)	0.703
positive	42 (73.7)	40 (59.7		50(67.6)	32 (64.0)	
ErbB1/HER1						
negative	50 (87.7)	62 (92.5)	0.544	65 (87.9)	47 (94.0)	0.358
positive	7 (12.3)	5 (7.5)		9 (12.2)	3 (6.0)	
ErbB3/HER3						
negative	15 (26.3)	22 (32.8)	0.440	24 (32.4)	13 (26.0)	0.549
positive	42 (73.7)	45 (67.2)		50 (67.6)	37 (74.0)	
ErbB4/HER4						
negative	21 (36.8)	15 (22.4)	0.112	24 (32.4)	12 (24.0)	0.323
positive	36 (63.2)	52 (77.6)		50 (67.6)	38 (76.0)	
ER						
negative				54 (73.0)	3 (6.0)	<0.001‡
positive				20 (270)	47 (94.0)	
PgR						
negative	54 (94.7)	20 (29.9)	<0.001‡			
positive	3 (5.3)	47 (70.1)				

*Abbreviations: HER1 – human epidermal growth factor receptor 1; HER3 – human epidermal growth factor receptor 3; HER4 – human epidermal growth factor receptor 4; luminal B – estrogen receptor (ER) and/or progesterone receptor (PgR)-positive and HER2 positive; HER2E – ER- and PgR-negative and HER2-positive intrinsic subtype; pHER2^{Y1248} – tyrosine 1248-phosphorylated human epidermal growth factor receptor 2.
†Bonferroni non-adjusted *P* values.
‡Significant *P* values (<Pc). Bonferroni correction for multiple comparisons Pc = 0.004 (0.05/12 - number of comparisons). Pearson chi square or Fisher exact test were used as appropriate.

Survival analyses

Kaplan-Meier analysis showed that patients with larger tumor size ($P=0.043$), higher histological grade ($P=0.005$) (Figure 3), and positive lymph nodes ($P<0.001$) were more likely to relapse after trastuzumab-based therapy (Figure 4). Nevertheless, after the Bonferroni correction only the effect of positive lymph node status remained significant ($P<P_c$ [0.05/12 = 0.004]). Patients with DP were in most cases younger than 60 years (67.6%), had negative hormone receptor status (ER – 55.9%; PgR – 67.6%), and HER2E intrinsic subtype (52.9%). Notably, 58.8% of patients with DP showed pHER2^{Y1248}-negative staining. In addition, the majority had HER1-negative (94.1%) and HER3-positive or HER4-postive status (67.6% and 79.4%, respectively, Table 4).

TABLE 4. The effect of clinicopathological variables on disease-free survival of HER2-positive breast cancer (BC) patients. The values are presented as frequencies and percentages*

	Disease progression		
	no	yes	$P^{\dagger}$
Age stratification (years)			0.535
<60	54 (60.0)	23 (67.6)	
>60	36 (40.0)	11 (32.4)	
Tumor size (cm)			0.066
<2.0	41 (45.6)	9 (26.5)	
>2.0	49 (54.4)	25 (73.5)	
Histological grade			0.008
II	46 (51,1)	8 (23.5)	
III	44 (48,9)	26 (76.5)	
ER			0.226
negative	38 (42.2)	19 (55.9)	
positive	52 (57.8)	15 (44.1)	
PgR			0.309
negative	51 (56.7)	23 (67.6)	
positive	39 (43.3)	11 (32.4)	
Intrinsic subtype			0.226
luminal B	54 (60.0)	16 (47.1)	
HER2E	36 (40.0)	18 (52.9)	
Lymph node status			0.001‡
negative	44 (48.9)	5 (14.7)	
positive	46 (51.1)	29 (85.3)	
pHER2^{Y1248}			0.001‡
negative	22 (24.4)	20 (58.8)	
positive	68 (75.6)	14 (41.2)	
HER1			0.509
negative	80 (88.9)	32 (94.1)	
positive	10 (11.1)	2 (5.9)	
HER3			0.826
negative	26 (28.9)	11 (32.4)	
positive	64 (71.1)	23 (67.6)	
HER4			0.269
negative	29 (32.2)	7 (20.6)	
positive	61 (67.8)	27 (79.4)	

*Abbreviations: HER1 – human epidermal growth factor receptor 1; HER3 – human epidermal growth factor receptor 3; HER4 – human epidermal growth factor receptor 4; luminal B – estrogen receptor (ER) and/or progesterone receptor (PgR)-positive and HER2 positive; HER2E – ER, and PgR-negative and HER2-positive intrinsic subtype; pHER2^{Y1248} – tyrosine 1248- phosphorylated human epidermal growth factor receptor 2.
†Bonferroni non-adjusted *P* values.
‡Significant *P* values (<Pc). Bonferroni correction for multiple comparisons Pc = 0.005 (0.05/11 - number of comparisons). Pearson chi square or Fisher exact test were used as appropriate.

pHER2^{Y1248}-negative status was significantly negatively associated with five-year DFS after trastuzumab treatment ($P<0.001$, Figure 4). The mean DFS of pHER2^{Y1248}-negative patients was 56.8 years, compared with 74.9 years in pHER2^{Y1248}-positive patients (Figure 4). Moreover, univariate Cox regression analysis revealed that the pHER2^{Y1248}-negative status was the most significant prognostic factor for a worse five-year DFS (HR 3.4, 95% CI 1.7-6.8, $P<0.001<P_c$ [0.05/11 = 0.005]) (Table 5). Independent prognostic power of pHER2^{Y1248}-negative status in predicting DFS was further confirmed with multivariate Cox regression analysis (HR 3.6, 95% CI 1.8-7.2, $P<0.001$).

The average DFS of pHER2^{Y1248}-negative patients older than 60 years, with higher histological tumor grade, a larger tumor, or positive lymph nodes was 54.8, 47.2, 46.2, and 44.9 months, respectively (Figure 5 and 6).

In univariate Cox regression analysis, positive lymph nodes (HR 4.6, 95% CI 1.8-11.9, $P=0.002<P_c$ [0.05/11 = 0.005]) and higher histological grade (HR 2.96, 95% CI 1.3-6.5, $P=0.007$) were both negatively associated with five-year DFS (Table 5). However, multivariate Cox regression analysis confirmed only positive lymph node status (HR 3.6, 95% CI 1.3-9.8, $P=0.014$) as an additional indicator of a worse five-year DFS (Table 5).

Univariate Cox regression analysis revealed that the intrinsic subtype, hormone receptor status, and HER3 and HER4 staining were not significantly related to DFS (Table 5). Nevertheless, when these biomarkers were stratified according to pHER2^{Y1248} status, Kaplan-Meier analysis showed significant differences in five-year DFS ($P<P_c$ [0.05/11 = 0.005]) (Figure 6). Thus, pHER2^{Y1248}-negative patients with HER2E intrinsic subtype, as well as those with HER1-negative staining (Figure 7) or negative hormone receptors (Figure 8) had a worse five-year

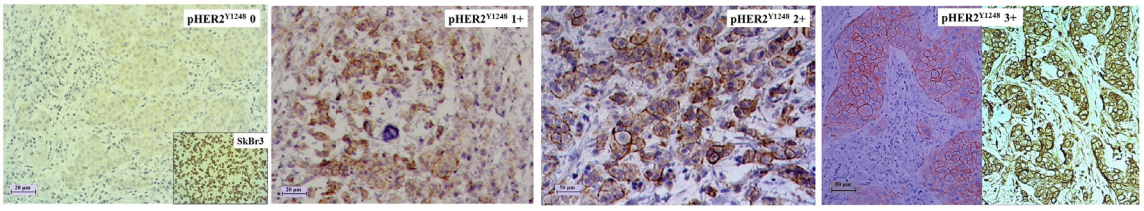


FIGURE 1. Immunohistochemical staining of tyrosine 1248-phosphorylated human epidermal growth factor receptor 2 (pHER2^{Y1248}). From left to right: pHER2Y^{1248} 0, none or weak membranous staining; square – positive control of SkBr3 breast cancer (BC) cell line cells (overexpresses Her2 [Neu/ErbB-2] gene product stained for pHER2^{Y1248}; pHER2^{Y1248} 1+, weak, fragmented membranous staining in >10% of tumor cells; pHER2^{Y1248} 2+, weak to moderate complete membrane staining in >10% of tumor cell, and pHER2^{Y1248} 3+, different variants of strong membranous staining in >10% of tumor cells.

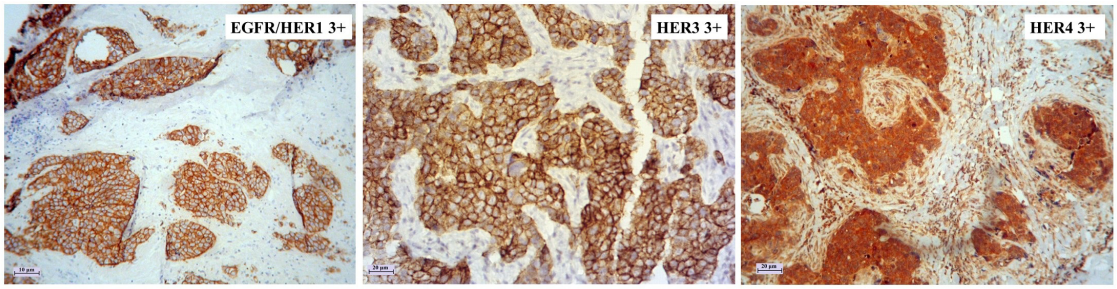
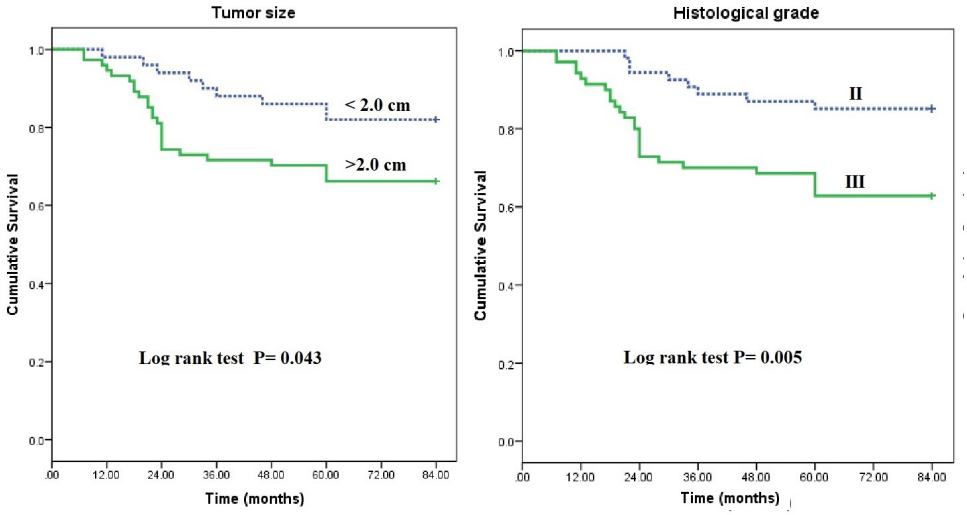


FIGURE 2. Immunohistochemical staining for human epidermal growth factor receptors (HER) analyzed in this study. Only strong immunohistochemical staining pattern is presented. From left to right: membranous EGFR/HER1 staining; membranous HER3 staining, and membranous/cytoplasmic HER4 staining.

TABLE 5. Predictors of five-year disease-free survival on univariate and multivariate analysis of breast cancer (BC) HER2-positive patients*

	Univariate		Multivariate	
Variable	HR (95% CI)	Cox P†	HR (95% CI)	Cox P§
pHER2^{Y1248} (negative vs positive)	3.4 (1.7-6.8)	<0.001‡	3.6 (1.8-7.2)	<0.001
Lymph node (positive vs negative)	4.6 (1.8-11.9)	0.002‡	3.6 (1.3-9.8)	0.014
Histological grade (III vs II)	2.96 (1.3-6.5)	0.007	2.0 (0.9-4.6)	0.116
Tumor size (≥2.0 cm vs <2.0 cm)	2.1 (1.0-4.6)	0.050		
Intrinsic subtypes (Luminal B vs HER2E)	0.6 (0.3-1.2)	0.168		
Age (<60 vs ≥60 years)	1.3 (0.6-2.6)	0.539		
ER (negative vs positive)	1.6 (0.8-3.2)	0.159		
PgR (negative vs positive)	1.6 (0.0-3.2)	0.213		
HER1 (negative vs positive)	1.8 (0.4-7.7)	0.405		
HER3 (positive vs negative)	0.9 (0.4-1.83)	0.755		
HER4 (positive vs negative)	1.7 (0.7-3.9)	0.220		

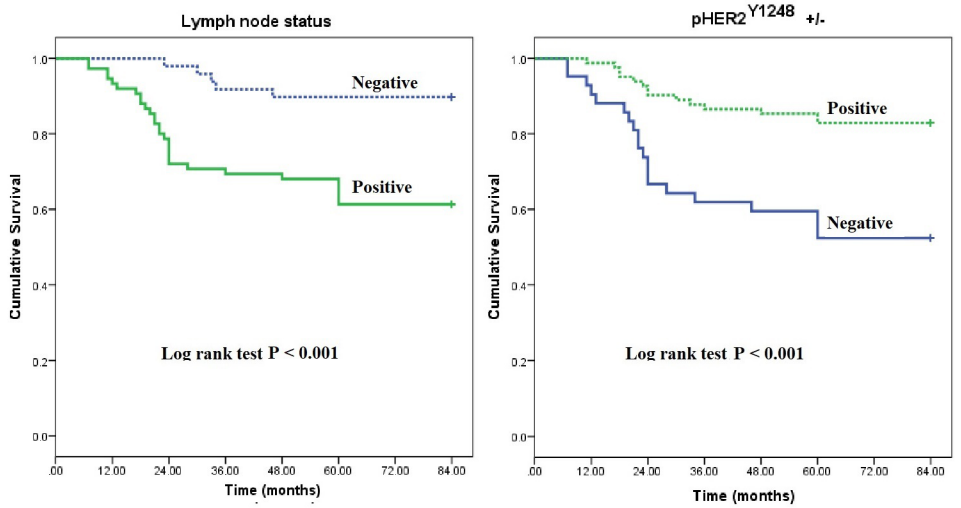
*Abbreviations: CI – confidence interval; HR – hazard ratio; pHER2^{Y1248} – tyrosine 1248–phosphorylated human epidermal growth factor receptor 2; HER1 – human epidermal growth factor receptor 1; HER3 – human epidermal growth factor receptor 3; HER4 – human epidermal growth factor receptor 4; Luminal B – estrogen receptor (ER) and/or progesterone receptor (PgR)-positive and HER2 positive; HER2E – ER, and PgR-negative, and HER2-positive intrinsic subtype; Cox P – *P* values for Cox regression analysis;
†Bonferroni non-adjusted *P* values.
‡Significant *P* values ($<P_c = 0.005$ in univariate Cox regression analysis). Bonferroni correction for multiple tests in univariate Cox regression analysis $P_c = 0.005$ (0.05/11– number of comparisons).
§Bonferroni correction not applicable.



Tumor size	*Mean time*	*95% Confidence Interval*	
		Lower Bound	*Upper Bound*
<20mm	75.26	69.75	80.77
>20mm	64.28	57.68	70.88
Overall	68.71	64.09	73.33

Histological grade	*Mean time*	*95% Confidence Interval*	
		Lower Bound	*Upper Bound*
II	76.57	71.65	81.50
III	62.64	55.72	69.56

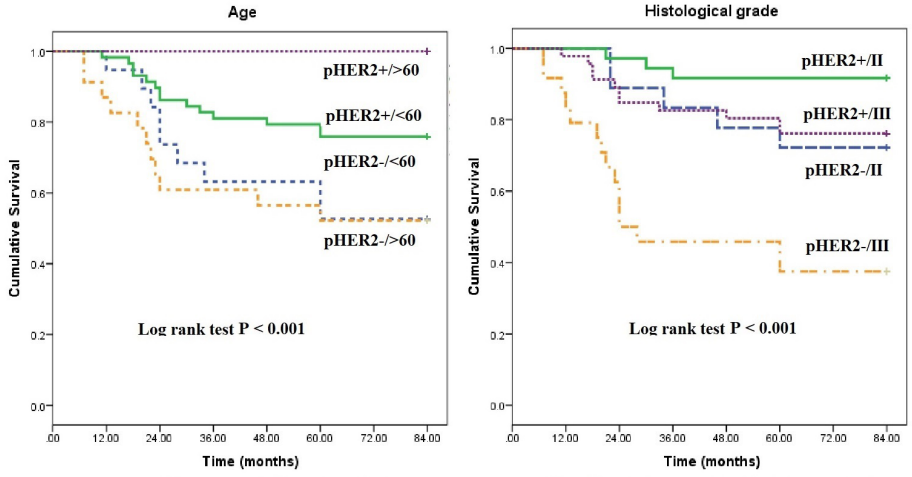
FIGURE 3. Kaplan-Meier estimates of five-year disease-free survival rate among breast cancer patients stratified according to tumor size (<2.0 cm vs >2.0 cm) and histological grade (II vs III).



Lymph node status	*Mean time*	*95% Confidence Interval*	
		Lower Bound	*Upper Bound*
Negative	78.82	74.46	83.17
Positive	62.11	55.43	68.78
Overall	68.71	64.09	73.33

pHER2^{Y1248}	*Mean time*	*95% Confidence Interval*	
		Lower Bound	*Upper Bound*
No	56.79	47.50	66.07
Yes	74.82	70.23	79.40

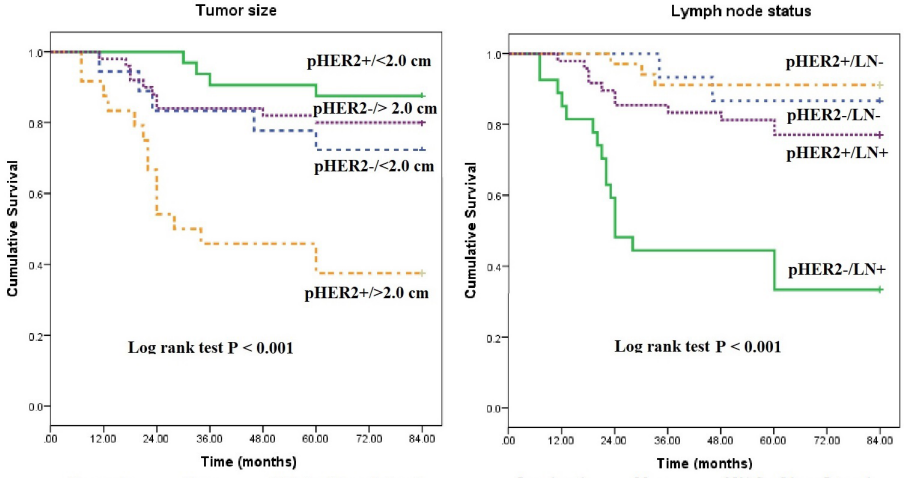
FIGURE 4. Kaplan-Meier estimates of five-year disease-free survival rate among breast cancer patients stratified according to lymph node status (LN- vs LN+) and pHER2^{Y1248}-positive and pHER2^{Y1248}-negative immunostaining pattern. Abbreviations: pHER2^{Y1248} – tyrosine 1248-phosphorylated human epidermal growth factor receptor 2.



Age	*Mean time*	*95% Confidence Interval*	
		Lower Bound	*Upper Bound*
pHER2-/<60	59.16	45.01	73.25
pHER2+/<60	71.02	64.60	77.43
pHER2-/>60	54.83	40.53	69.12
pHER2+/>60	84.00	84.00	84.00
Overall	68.71	64.09	73.33

Histological grade	*Mean time*	*95% Confidence Interval*	
		Lower Bound	*Upper Bound*
pHER2-/II	70.89	60.49	81.28
pHER2+/II	79.42	74.42	84.42
pHER2-/III	46.21	33.50	58.92
pHER2+/III	71.22	64.22	78.22

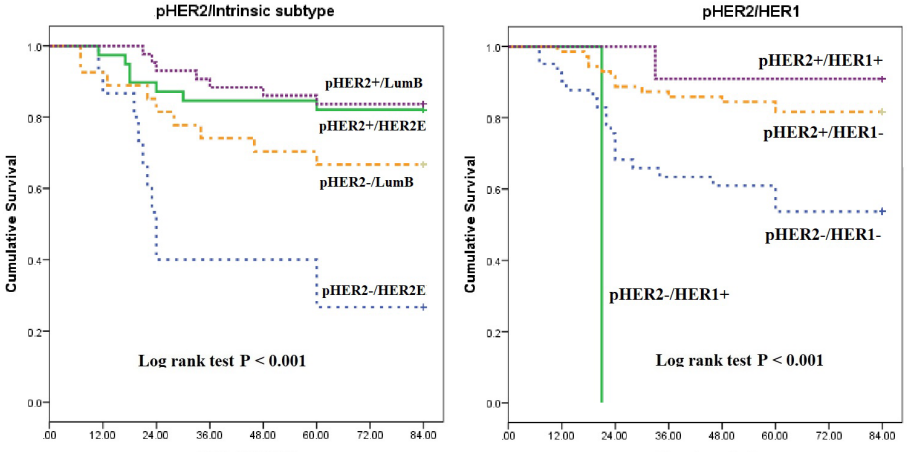
FIGURE 5. Kaplan-Meier estimates of five-year disease-free survival rate of breast cancer patients with positive and negative tyrosine 1248-phosphorylated human epidermal growth factor receptor 2 (pHER2^{Y1248}) immunohistochemical status stratified according to age subgroups at surgery (<60 years vs >60 years) and tumor histological grade (II vs III).



Tumor size	*Mean time*	*95% Confidence Interval*	
		Lower Bound	*Upper Bound*
pHER2-/<2.0 cm	69.56	57.92	81.19
pHER2+/<2.0 cm	78.47	73.19	83.74
pHER2-/> 2.0 cm	47.21	34.81	59.61
pHER2+/>2.0 cm	72.48	65.84	79.12
Overall	68.71	64.09	73.33

Lymph node status	*Mean time*	*95% Confidence Interval*	
		Lower Bound	*Upper Bound*
pHER2-/LN-	78.13	70.48	85.78
pHER2-/LN+	44.93	33.32	56.53
pHER2+/LN-	79.12	73.83	84.41
pHER2+/LN+	71.77	65.03	78.51

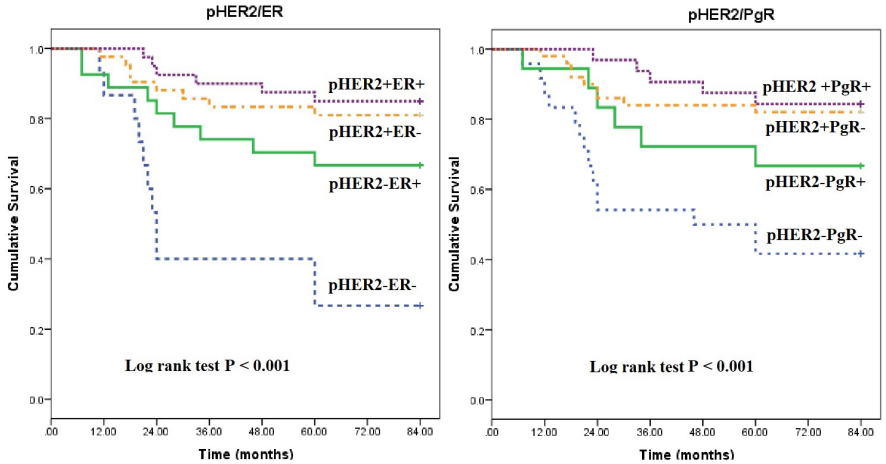
FIGURE 6. Kaplan-Meier estimates of five-year disease-free survival rate of breast cancer patients with positive and negative tyrosine 1248-phosphorylated human epidermal growth factor receptor 2 (pHER2^{Y1248}) immunohistochemical status stratified according to tumor size (<2.0 cm vs >2.0 cm) and lymph node status (LN+ vs LN-).



pHER2-Intrinsic subtype	*Mean time*	*95% Confidence Interval*	
		Lower Bound	*Upper Bound*
pHER2-/HER2E	42.13	27.57	56.70
pHER2+/HER2E	73.49	66.15	80.82
pHER2-/LumB	64.93	54.12	75.74
pHER2+/LumB	76.02	70.38	81.67
Overall	68.71	64.09	73.33

pHER2-HER1	*Mean time*	*95% Confidence Interval*	
		Lower Bound	*Upper Bound*
pHER2-/HER1-	57.66	48.30	67.01
pHER2-/HER1+	21.00	21.00	21.00
pHER2+/HER1-	74.11	69.01	79.21
pHER2+/HER1+	79.36	70.70	88.023

FIGURE 7. Kaplan-Meier estimates of five-year disease-free survival rate of breast cancer patients with positive and negative tyrosine 1248-phosphorylated human epidermal growth factor receptor 2 (pHER2^{Y1248}) immunohistochemical status stratified according to tumor intrinsic subtype (LumB vs HER2E), and HER1 immunohistochemical staining (HER1- vs HER1+). Abbreviations: LumB – estrogen receptor (ER)- and/or progesterone receptor (PgR)-positive and HER2-positive; HER2E – ER- and PgR-negative, and HER2-positive intrinsic subtype.



pHER2-ER	*Mean time*	*95% Confidence Interval*	
		Lower Bound	*Upper Bound*
pHER2-ER-	42.13	27.57	56.70
pHER2-ER+	64.93	54.12	75.74
pHER2+ER-	73.10	66.09	80.16
pHER2+ER+	76.63	70.91	82.34
Overall	68.71	64.09	73.33

pHER2-PgR	*Mean time*	*95% Confidence Interval*	
		Lower Bound	*Upper Bound*
pHER2-PgR-	50.08	37.56	62.61
pHER2-PgR+	65.72	53.04	78.41
pHER2+PgR-	73.34	66.84	79.84
pHER2 +PgR+	77.13	71.32	82.93

FIGURE 8. Kaplan-Meier estimates of five-year disease-free survival rate of breast cancer patients with positive and negative tyrosine 1248-phosphorylated human epidermal growth factor receptor 2 (pHER2^{Y1248}) immunohistochemical status stratified according to ER (ER- vs ER+) and PgR (PgR- vs PgR+) immunohistochemical staining. Abbreviations: ER – estrogen receptor, PgR – progesterone receptor.

DFS. The same was true for pHER2^{Y1248}-negative patients with the coexpression of HER3+ or HER4+ (Figure 9).

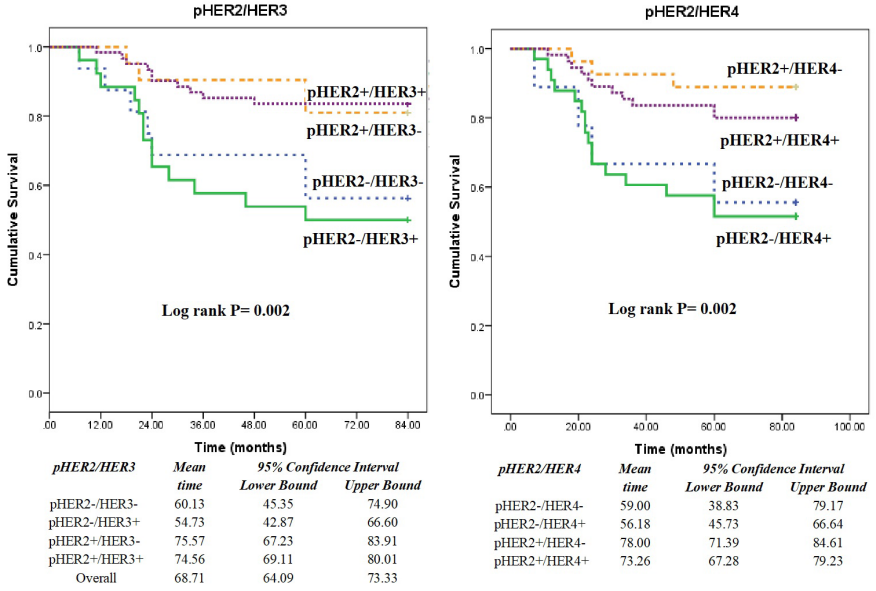
DISCUSSION

In our study, pHER2^{Y1248} predicted sensitivity to trastuzumab and a better five-year DFS regardless of any other prognostic parameter. Additionally, pHER2^{Y1248}-negative and lymph node-positive status were the only negative prognostic factors for five-year DFS of HER2-positive BC patients. Furthermore, non-phosphorylated HER2^{Y1248} was a strong predictor of resistance and poor five-year DFS in combination with any clinicopathological parameter: in patients older than 60 years or those with positive lymph nodes, larger tumor size, or higher histological stage. This was especially true in patients with HER2E-type tumor or those negative for EGFR/HER1 and/or positive for HER3 or HER4.

The expression of pHER2^{Y1248} receptors was identified in 66.1% of our patients, consistent with 68% of HER2-positive BC cases in the report by Cicenas et al (13). A relatively high expression rate of pHER2Y^{1248} in HER2-positive BC was also found by other researchers (14-19). However, smaller rates (12%-38.2%) were also reported (20-27). The discrepancies in pHER2^{Y1248} expression might be explained by differences in assays sensitivity, scoring systems, cut-off values used for the evaluation of pHER2 expression, and likely difference in the degree of phosphorylation/activation of HER2 in biologically distinct BC patient cohorts. Thus, for example, HER2 overexpression with concomitant pHER2^{Y1248}-positive status was considerably less common in ER+ BCs than previously believed (28).

Taniyama et al (19) showed pHER2^{Y1248} expression to be highly specific for HER2 gene amplification, advanced tumor stage, and poor DFS of patients with invasive ductal BC. Furthermore, the total level of pHER2^{Y1248} is considered to be physiologically more important than the overall number of HER2 present in the cancer tissue (20). Importantly, not all BC patients with pHER2^{Y1248} also overexpressed HER2, which indicates signaling activation distinct from the classical pathway involving homo-dimerization and hetero-dimerization with other ErbB family members (13,29). Previous studies also indicate that phosphorylation of HER2 at Y1248 occurs only when HER2 is dimerized and activated irrespective of its overexpression status (11,24).

Phosphorylation of HER2 leading to its activation is closely associated with subsequent signaling transduction to its downstream targets that mediate cellular proliferation,



pHER2/HER3	Mean time	95% Confidence Interval Lower Bound	Upper Bound
pHER2-/HER3-	60.13	45.35	74.90
pHER2-/HER3+	54.73	42.87	66.60
pHER2+/HER3-	75.57	67.23	83.91
pHER2+/HER3+	74.56	69.11	80.01
Overall	68.71	64.09	73.33

pHER2/HER4	Mean time	95% Confidence Interval Lower Bound	Upper Bound
pHER2-/HER4-	59.00	38.83	79.17
pHER2-/HER4+	56.18	45.73	66.64
pHER2+/HER4-	78.00	71.39	84.61
pHER2+/HER4+	73.26	67.28	79.23

FIGURE 9. Kaplan-Meier estimates of five-year disease-free survival rate of breast cancer (BC) patients with positive and negative tyrosine 1248-phosphorylated human epidermal growth factor receptor 2 (pHER2^{Y1248}) immunohistochemical status stratified according to HER3 (HER3- vs HER3+) and HER4 (HR4- vs HR4+) immunohistochemical staining. Abbreviations: HER – epidermal growth factor receptor.

migration, or adhesion, thus profoundly affecting DP and overall prognosis (30-38).

In our study, patients with positive pHER2^{Y1248} had a nearly three times lower risk of DP after trastuzumab-based treatment and a better five-year DFS compared with patients with negative pHER2^{Y1248}, of whom 58% relapsed.

Similarly, Hudelist et al (39) reported that pHER2^{Y1248}-positive staining was the only covariate predicting the benefit of trastuzumab-based treatment in metastatic BC patients exhibiting moderate or strong HER2 overexpression (39). Notably, in their cohort, the progression-free survival to trastuzumab-based treatment was more than doubled in pHER2^{Y1248}-positive compared with pHER2^{Y1248}-negative BC (39).

Better response of pHER2^{Y1248}-positive BC to trastuzumab-based therapy was also reported by Giuliani et al (27). Besides, Dokmanovic et al (40) reported that pHER2^{Y1248}-positive staining in HER2-positive BC correlated with increased trastuzumab response in the neoadjuvant settings. Notably, the majority of the patients with DP or residual disease after neoadjuvant trastuzumab treatment were pHER2^{Y1248}-negative, whereas 4/5 patients with complete or near-complete pathological remission were pHER2^{Y1248}-positive (40).

A trend toward increased sensitivity to trastuzumab treatment was also confirmed in experiments on HER2-overexpressing BC cell lines (9). In the study by Ginestier et al (9), the majority of cell lines (8/10) sensitive to trastuzumab treatment were pHER2^{Y1248}-positive, while 4/6 resistant cell lines were either weakly positive or negative. Furthermore, Diemeier et al (41) reported that the dominant growth-inhibitory effect of trastuzumab on trastuzumab-sensitive BT474 and SK-BR-3 BC cells lines was associated with its ability to induce HER2 phosphorylation at Y1248.

Contrary to this, Kurebayashi et al (18) reported a worse prognostic effect of pHER2^{Y1248} in HER2-positive BC patients treated with trastuzumab and chemotherapy. They hypothesized that the worse outcome of such patients might be related to the inability of trastuzumab to inhibit DP through ligand-dependent HER2-HER3 hetero-dimerization (42-44).

Interestingly, Cheng et al (46) found no association between pHER2^{Y1248} and the response to trastuzumab-containing neoadjuvant therapy in the pre-surgical setting. No association of pHER2^{Y1248} and trastuzumab treatment of HER2-positive BC patients was also reported by Dębska-Szmich et al (26). In other studies, pHER2^{Y1248}-positive staining was associated with a worse prognosis in primary, treatment-naive BC patients (13,14,16,20,24,47,48).

In our study, the majority of HER2-positive patients with DP exhibited hormone receptor-negative, EGFR/HER1-negative immunostaining, and positive HER3 and/or HER4 status. In a study by DiGiovanna et al (14), pHER2^{Y1248} expression was significantly associated with a positive expression of EGFR/HER1. A worse outcome was observed in patients who were either positive for all three biomarkers examined (pHER2^{Y1248}, EGFR/HER1, and HER2) or positive only for EGFR/HER1 and HER2 but negative for pHER2^{Y1248} (14). Significantly higher expression of pHER2^{Y1248} among patients with high EGFR/HER1 levels was also reported by Cicenas et al (13). However, despite the positive correlation between the pHER2^{Y1248}-positive status and EGFR/HER1, its overall expression did not differ between pHER2^{Y1248}-positive and pHER2^{Y1248}-negative patients. In addition, pHER2^{Y1248} expression positively correlated with HER2 and inversely correlated with hormone receptors, HER3, and HER4. Hormone receptors and HER4 levels were significantly lower in patients with higher pHER2^{Y1248} (13). Contrary to these reports, Kurebayashi et al (18) reported no significant correlation of pHER2^{Y1248} with EGFR/HER1 and HER4 expression in HER2-positive BC patients. However, pHER2^{Y1248} significantly correlated with HER2 and HER3 expression (18).

In our study, a worse five-year DFS was detected in patients with pHER2^{Y1248}-negative and either hormone receptor-negative or HER1-negative status and in patients with pHER2^{Y1248}-negative immunostaining co-expressed with positive HER3 or HER4. Our results suggest that in such cases phosphorylation occurred at other phosphorylation sites and heterodimerization activated other signaling pathways.

There are several limitations to our study. The sample size was moderate, encompassing BC patients with specific biomarker signatures. Second, the majority of BC in our patient cohort exhibited a HercepTest score of 3+, while the number of HER2 equivocal (2+) patients was limited. Third, all patients received adjuvant trastuzumab therapy so future studies should assess the prognostic value of pHER2^{Y1248} in the neoadjuvant settings.

In conclusion, our results indicate that the pHER2^{Y1248}-negative status of HER2-overexpressing BC repre-

sents a strong independent predictor of tumor resistance to trastuzumab-based therapy and poor five-year DFS rate irrespective of other biomarkers and clinicopathological variables tested. Further studies are needed to investigate the predictive value of the pHER2^{Y1248} in a larger cohort of HER2-positive BC patients.

Funding None.

Ethical approval given by the Ethics Committee of the University Hospital for Tumors (EP-15506/11-6).

Declaration of authorship SR and FK conceived and designed the study; all authors acquired the data; all authors analyzed and interpreted the data; SR and FP drafted the manuscript; all authors critically revised the manuscript for important intellectual content; all authors gave approval of the version to be submitted; all authors agree to be accountable for all aspects of the work.

Competing interests All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

References

1 Hanna WM, Slodkowska E, Lu FI, Nafisi H, Nofech-Mozes S. Comparative analysis of human epidermal growth factor receptor 2 testing in breast cancer according to 2007 and 2013 American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations. J Clin Oncol. 2017;35:3039-45. Medline:28445098 doi:10.1200/JCO.2016.70.5319

2 Woo JW, Lee K, Chung YR, Jang MH, Ahn S, Park SY. The updated 2018 American Society of Clinical Oncology/College of American Pathologists guideline on human epidermal growth factor receptor 2 interpretation in breast cancer: comparison with previous guidelines and clinical significance of the proposed in situ hybridization groups. Hum Pathol. 2020;98:10-21. Medline:32027910 doi:10.1016/j.humpath.2020.01.003

3 Wolff AC, Hammond MEH, Allison KH, Harvey BE, Mangu PB, Bartlett JMS, et al. Human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update. J Clin Oncol. 2018;36:2105-22. Medline:29846122 doi:10.1200/JCO.2018.77.8738

4 Wang J, Xu B. Targeted therapeutic options and future perspectives for HER2-positive breast cancer. Signal Transduct Target Ther. 2019;4:34. Medline:31637013 doi:10.1038/s41392-019-0069-2

5 Perez EA, Romond EH, Suman VJ, Jeong JH, Sledge G, Geyer CE Jr, et al. Trastuzumab plus adjuvant chemotherapy for human epidermal growth factor receptor 2-positive breast cancer: planned joint analysis of overall survival from NSABP B-31 and NCCTG N9831. J Clin Oncol. 2014;32:3744-52. Medline:25332249 doi:10.1200/JCO.2014.55.5730

6 Watson SS, Dane M, Chin K, Tatarova Z, Liu M, Liby T, et al. Microenvironment-mediated mechanisms of resistance to HER2 inhibitors differ between HER2+ breast cancer subtypes. Cell Syst. 2018;6:329-342.e6. Medline:29550255 doi:10.1016/j.cels.2018.02.001

7 Cvetanovic A, Pejcic I, Zivkovic N, Krtinic D, Kostic M, Popovic L. Do we really know how to overcome trastuzumab resistance in hormone sensitive metastatic breast cancer? J BUON. 2019;24:516-21. Medline:31127999

8 Ghosh R, Narasanna A, Wang SE, Liu S, Chakrabarty A, Balko JM, et al. Trastuzumab has preferential activity against breast cancers driven by HER2 homodimers. Cancer Res. 2011;71:1871-82. Medline:21324925 doi:10.1158/0008-5472.CAN-10-1872

9 Ginestier C, Adélaïde J, Gonçalvès A, Repellini L, Sircoulomb F, Letessier A, et al. ERBB2 phosphorylation and trastuzumab sensitivity of breast cancer cell lines. Oncogene. 2007;26:7163-9. Medline:17525746 doi:10.1038/sj.onc.1210528

10 Ramić S, Asić K, Balja MP, Paić F, Benković V, Knežević F. Correlation of phosphorylated HER2 with clinicopathological characteristics and efficacy of trastuzumab treatment for breast cancer. Anticancer Res. 2013;33:2509-15. Medline:23749902

11 Burguin A, Furrer D, Ouellette G, Jacob S, Diorio C, Durocher F. Trastuzumab effects depend on HER2 phosphorylation in HER2-negative breast cancer cell lines. PLoS One. 2020;15:e0234991. Medline:32584853 doi:10.1371/journal.pone.0234991

12 Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, et al; American Society of Clinical Oncology. College of American Pathologists. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol. 2013;31:3997-4013. Medline:24101045 doi:10.1200/JCO.2013.50.9984

13 Cicenas J, Urban P, Küng W, Vuaroqueaux V, Labuhn M, Wight E, et al. 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. 2006;42:636-45. Medline:16414259 doi:10.1016/j.ejca.2005.11.012

14 DiGiovanna MP, Stern DF, Edgerton SM, Whalen SG, Moore D II, Thor AD. Relationship of epidermal growth factor receptor expression to ErbB-2 signaling activity and prognosis in breast cancer patients. J Clin Oncol. 2005;23:1152-60. Medline:15718311 doi:10.1200/JCO.2005.09.055

15 DiGiovanna MP, Chu P, Davison TL, Howe CL, Carter D, Claus EB, et al. Active signaling by HER-2/neu in a subpopulation of HER-2/neu-overexpressing ductal carcinoma in situ: clinicopathological correlates. Cancer Res. 2002;62:6667-73. Medline:12438265

16 Hayashi N, Iwamoto T, Gonzalez-Angulo AM, Ferrer-Lozano J, Lluch A, Niikura N, et al. Prognostic impact of phosphorylated HER-2 in HER-2+ primary breast cancer. Oncologist. 2011;16:956-65. Medline:21712485 doi:10.1634/theoncologist.2010-0409

17 Eppenberger-Castori S, Kueng W, Benz C, Caduff R, Varga Z, Bannwart F, et al. Prognostic and predictive significance of ErbB-2 breast tumor levels measured by enzyme immunoassay. J Clin Oncol. 2001;19:645-56. Medline:11157014 doi:10.1200/JCO.2001.19.3.645

18 Kurebayashi J, Kanomata N, Yamashita T, Shimo T, Mizutoh A, Moriya T, et al. Prognostic value of phosphorylated HER2 in HER2-positive breast cancer patients treated with adjuvant trastuzumab. Breast Cancer. 2015;22:292-9. Medline:23749689 doi:10.1007/s12282-013-0478-y

19 Taniyama K, Ishida K, Toda T, Motoshita J, Kuraoka K, Saito A, et al. Tyrosine1248-phosphorylated HER2 expression and HER2 gene amplification in female invasive ductal carcinomas. Breast Cancer. 2008;15:231-40. Medline:18264744 doi:10.1007/s12282-007-0026-8

20 Thor AD, Liu S, Edgerton S, Moore D, Kasowitz KM, Benz CC, et al. Activation (tyrosine phosphorylation) of ErbB-2 (HER-2/neu): a study of incidence and correlation with outcome in breast cancer. J Clin Oncol. 2000;18:3230-9. Medline:10986055 doi:10.1200/JCO.2000.18.18.3230

21 DiGiovanna MP, Carter D, Flynn SD, Stern DF. Functional assay for HER-2/neu demonstrates active signaling in a minority of HER-2/neu-overexpressing invasive human breast tumours. Br J Cancer. 1996;74:802-6. Medline:8795585 doi:10.1038/bjc.1996.439

22 Ouyang X, Gulliford T, Doherty A, Huang GC, Epstein RJ. Detection of ErbB2 oversignalling in a majority of breast cancers with phosphorylation-state-specific antibodies. Lancet. 1999;353:1591-2. Medline:10334266 doi:10.1016/S0140-6736(99)01095-8

23 Ouyang X, Gulliford T, Huang GC, Harper-Wynne C, Shousha S, Epstein RJ. Multisite phosphotyping of the ErbB-2 oncoprotein in human breast cancer. Mol Diagn. 2001;6:17-25. Medline:11257208 doi:10.2165/00066982-200106010-00003

24 Frogne T, Laenkholm AV, Lyng MB, Henriksen KL, 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. 2009;11:R11. Medline:19239686 doi:10.1186/bcr2230

25 Hudelist G, Köstler WJ, Czerwenka K, Kubista E, Attems J, Müller R, et al. Her-2/neu and EGFR tyrosine kinase activation predict the efficacy of trastuzumab-based therapy in patients with metastatic breast cancer. Int J Cancer. 2006;118:1126-34. Medline:16161043 doi:10.1002/ijc.21492

26 Dębska-Szmich S, Kusińska R, Czernek U, Szydłowska-Pazera K, Habib-Lisik M, Piekarski JH, et al. Prognostic value of HER3, PTEN and p-HER2 expression in patients with HER2 positive breast cancer. Postepy Hig Med Dosw. 2015;69:586-97. Medline:25983297

27 Giuliani R, Durbecq V, Di Leo A, Paesmans M, Larsimont D, Leroy JY, et al. 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 (MBC). Eur J Cancer. 2007;43:725-35. Medline:17251007 doi:10.1016/j.ejca.2006.11.019

28 Singer CF, Gschwantler-Kaulich D, Fink-Retter A, Pfeiler G, Walter I, Hudelist G, et al. HER2 overexpression and activation, and tamoxifen efficacy in receptor-positive early breast cancer. J Cancer Res Clin Oncol. 2009;135:807-13. Medline:19034514 doi:10.1007/s00432-008-0516-x

29 Wulfkuhle JD, Berg D, Wolff C, Langer R, Tran K, Illi J, et al. Molecular analysis of HER2 signaling in human breast cancer by functional protein pathway activation mapping. Clin Cancer Res. 2012;18:6426-35. Medline:23045247 doi:10.1158/1078-0432.CCR-12-0452

30 Elster N, Collins DM, Toomey S, Crown J, Eustace AJ, Hennessy BT. HER2-family signaling mechanisms, clinical implications and targeting in breast cancer. Breast Cancer Res Treat. 2015;149:5-15. Medline:25542271 doi:10.1007/s10549-014-3250-x

31 Eccles SA. The epidermal growth factor receptor/Erb-B/HER family in normal and malignant breast biology. Int J Dev Biol. 2011;55:685-96. Medline:22161825 doi:10.1387/ijdb.113396se

32 Hsu JL, Hung MC. The role of HER2, EGFR, and other receptor tyrosine kinases in breast cancer. Cancer Metastasis Rev. 2016;35:575-88. Medline:27913999 doi:10.1007/s10555-016-9649-6

33 Wang Z. ErbB receptors and cancer. Methods Mol Biol. 2017;1652:3-35. Medline:28791631 doi:10.1007/978-1-4939-7219-7_1

34 de Melo Gagliato D, Jardim DL, Marchesi MS, Hortobagyi GN. Mechanisms of resistance and sensitivity to anti-HER2 therapies in HER2+ breast cancer. Oncotarget. 2016;7:64431-46. Medline:26824988 doi:10.18632/oncotarget.7043

35 Appert-Collin A, Hubert P, Crémel G, Bennasroune A. Role of ErbB receptors in cancer cell migration and invasion. Front Pharmacol. 2015;6:283. Medline:26635612 doi:10.3389/fphar.2015.00283

36 Nahta R, Yu D, Hung MC, Hortobagyi GN, Esteva FJ. Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. Nat Clin Pract Oncol. 2006;3:269-80. Medline:16683005 doi:10.1038/ncponc0509

37 Khurshid R, Saleem M. Gul-e-Raana, Akhthar MS. Phosphorylation sites of HER2/c-erbB-2: role in cell growth and in disease. Acta Biochim Pol. 2014;61:699-703. Medline:25399009 doi:10.18388/abp.2014_1833

38 Ferreira PMP, Pessoa C. Molecular biology of human epidermal receptors, signaling pathways and targeted therapy against cancers: New evidences and old challenges. Braz J Pharm Sci. 2017;53:e16076. doi:10.1590/s2175-97902017000216076

39 Hudelist G, Köstler WJ, Attems J, Czerwenka K, Müller R, Manavi M, et al. 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. 2003;89:983-91. Medline:12966413 doi:10.1038/sj.bjc.6601160

40 Dokmanovic M, Wu Y, Shen Y, Chen J, Hirsch DS, Wu WJ. Trastuzumab-induced recruitment of Csk-homologous kinase (CHK) to ErbB2 receptor is associated with ErbB2-Y1248 phosphorylation and ErbB2 degradation to mediate cell growth inhibition. Cancer Biol Ther. 2014;15:1029-41. Medline:24835103 doi:10.4161/cbt.29171

41 Diermeier S, Horváth G, Knuechel-Clarke R, Hofstaedter F, Szöllosi J, Brockhoff G. Epidermal growth factor receptor coexpression modulates susceptibility to Herceptin in HER2/neu overexpressing breast cancer cells via specific erbB-receptor interaction and activation. Exp Cell Res. 2005;304:604-19. Medline:15748904 doi:10.1016/j.yexcr.2004.12.008

42 Vu T, Claret FX. Trastuzumab: updated mechanisms of action and resistance in breast cancer. Front Oncol. 2012;2:62. Medline:22720269 doi:10.3389/fonc.2012.00062

43 Wehrman TS, Raab WJ, Casipit CL, Doyonnas R, Pomerantz JH, Blau HM. A system for quantifying dynamic protein interactions defines a role for Herceptin in modulating ErbB2 interactions. Proc Natl Acad Sci U S A. 2006;103:19063-868. Medline:17148612 doi:10.1073/pnas.0605218103

44 Watanabe S, Yonesaka K, Tanizaki J, Nonagase Y, Takegawa N, Haratani K, et al. Targeting of the HER2/HER3 signaling axis overcomes ligand-mediated resistance to trastuzumab in HER2-positive breast cancer. Cancer Med. 2019;8:1258-68. Medline:30701699 doi:10.1002/cam4.1995

45 Zhang Y. The root cause of drug resistance in HER2-positive breast cancer and the therapeutic approaches to overcoming the resistance. Pharmacol Ther. 2021;218:107677. Medline:32898548 doi:10.1016/j.pharmthera.2020.107677

46 Cheng H, Bai Y, Sikov W, Sinclair N, Bossuyt V, Abu-Khalaf MM, et al. Quantitative measurements of HER2 and phospho-HER2 expression: correlation with pathologic response to neoadjuvant chemotherapy and trastuzumab. BMC Cancer. 2014;14:326. Medline:24885187 doi:10.1186/1471-2407-14-326

47 Ouyang X, Gulliford T, Huang GC, Harper-Wynne C, Shousha S, Epstein RJ. Multisite phosphotyping of the ErbB-2 oncoprotein in human breast cancer. Mol Diagn. 2001;6:17-25. Medline:11257208 doi:10.2165/00066982-200106010-00003

48 Eppenberger-Castori S, Kueng W, Benz C, Caduff R, Varga Z, Bannwart F, et al. Prognostic and predictive significance of ErbB-2 breast tumor levels measured by enzyme immunoassay. J Clin Oncol. 2001;19:645-56. Medline:11157014 doi:10.1200/JCO.2001.19.3.645