Immunohistochemical Features Associated with Sensitivity to Lapatinib-plus-Capecitabine and Resistance to Trastuzumab in HER2-positive Breast Cancer

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Abstract. *Aim: To identify immunohistochemical (IHC) features associated with sensitivity to lapatinib-plus-capecitabine (LX) and resistance to trastuzumab in human epidermal growth factor receptor (HER)-2-positive metastatic breast cancer. Patients and Methods: Expression levels of estrogen receptor, progesterone receptor, epidermal growth factor receptor, HER2, HER3/phosphorylated HER3 (pHER3), phosphatase and tensin homolog, thymidylate synthase (TYMS), and thymidine phosphorylase by IHC were compared between patients treated with LX following trastuzumab failure. Results: In 35 patients, HER2 was the only biomarker associated with LX treatment outcomes. A high HER2 level was associated with significantly longer survival and a tendency towards longer time-to-progression and higher response rates. Acquisition of trastuzumab resistance was associated with higher pHER3 and TYMS expression. Elevated pHER3 was predictive of superior treatment outcomes. Conclusion: Up-regulation of pHER3 and TYMS was associated with trastuzumab resistance. High HER2 and increased pHER3 IHC levels correlated with favourable LX treatment outcomes in patients with HER2-positive metastatic breast cancer.*

Trastuzumab, a humanized monoclonal antibody directed against the ectodomain of human epidermal growth factor receptor (HER)-2, in combination with chemotherapy improves overall survival (OS) in patients with HER2-positive breast cancer (1-3). However, only one-third of patients with HER2-positive metastatic breast cancer (MBC) show an initial response, and disease in most patients eventually becomes trastuzumab-resistant within one year (3). Lapatinib is a small-molecule tyrosine kinase inhibitor of the epidermal growth factor receptor (EGFR) and HER2. Lapatinib-plus-capecitabine significantly prolonged time-to-disease progression (TTP) compared with capecitabine monotherapy in patients with HER2-positive MBC that had progressed after treatment with an anthracycline, a taxane, and trastuzumab (4). Lapatinib plus capecitabine therapy is approved for HER2-positive breast cancer, but clinical benefit is limited to <30% of patients with trastuzumab-resistant disease (4).

Therefore, this study aimed to identify biomarkers associated with sensitivity to lapatinib plus capecitabine. Nine molecular targets, which have been implicated in sensitivity to lapatinib plus capecitabine and resistance to trastuzumab in HER2-positive MBC were evaluated: HER family members [EGFR, HER2, HER3, and phosphorylated HER3 (pHER3)]; molecules that may interact with HER signalling [oestrogen receptor (ER), progesterone receptor (PR), and phosphatase and tensin homolog (PTEN)]; and key enzymes involved in 5-fluorouracil metabolism [thymidylate synthase (TYMS) and thymidine phosphorylase (TYMP)]. We determined the association of immunohistochemical (IHC) expression levels of these targets with lapatinib plus capecitabine treatment outcome. Additionally, tumour tissues were classified as trastuzumab-resistant or trastuzumab-sensitive, and the IHC features associated with trastuzumab resistance were evaluated.

Patients and Methods

Patients and treatment. The study cohort comprised of patients with advanced breast cancer enrolled in the GlaxoSmithKline Lapatinib Expanded Access Program (LEAP) at Seoul National University

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Hospital between February 2007 and April 2008 and who agreed to participate in the pharmacogenomics study. The inclusion criteria were ≥18 years of age with a life expectancy of eight weeks or more; locally advanced or MBC that had progressed after treatment regimens consisting of an anthracycline, a taxane, and trastuzumab; HER2 overexpression [IHC 3+ or gene amplification by fluorescence *in situ* hybridization (FISH)]; Eastern Cooperative Oncology Group performance status of 0-2; cardiac ejection fraction within the normal range; adequate renal, hepatic, and hematologic function; no contraindication to lapatinib plus capecitabine; and at least one measurable lesion. Patients previously treated with lapatinib were excluded, whereas prior exposure to capecitabine was permitted.

Treatment consisted of 1,250 mg/day lapatinib and 2000 mg/m^2 capecitabine in two divided doses on days 1 to 14 of a 21-day cycle. Treatment was continued until disease progression, intolerable toxicity, or patient consent withdrawal. Treatment response was evaluated every six weeks according to the Response Evaluation Criteria in Solid Tumors (version 1.0) (5). Complete or partial responses (PRs) were confirmed by computed tomographic scans taken at least four weeks apart. Brain metastasis was evaluated using magnetic resonance imaging. Clinical data were collected and responses were evaluated prospectively. Written informed consent for LEAP and the pharmacogenomics study was obtained from all patients before study entry. The study protocol was reviewed and approved by the Institutional Review Board at Seoul National University Hospital (H-0701-065-196). Declaration of Helsinki recommendations for biomedical research involving human subjects were also followed.

Collection of tumor tissues. Pre-treatment biopsies of trastuzumab-resistant tumours (tumours that had progressed during trastuzumab therapy) were performed on patients who agreed to undergo the procedure, and collected tissues were used for the biomarker study. For the other patients, archival tumour tissues were used. In patients with multiple archival tumour tissues, the most recent tissue sample was selected. All patients were investigated for the presence of additional paired trastuzumab-resistant and trastuzumab-sensitive (tissues collected before initiation of trastuzumab) tumour tissues. To identify the IHC features associated with trastuzumab resistance, all tumour tissues collected were classified as trastuzumab-resistant and sensitive and IHC staining was compared between them.

IHC staining and interpretation. Hematoxylin and eosin staining was performed to confirm the presence of tumour. Antigen retrieval was performed using the appropriate method for each antibody. Samples were immunostained with the following antibodies according to the manufacturers' instructions: anti-ER (1:100; 1D5; Dako, Glostrup, Denmark), anti-PR (1:100; 636; Dako), anti-EGFR (1:100; 31G7; Zymed, San Francisco, CA, USA), anti-HER2 (1:200; A0485; Dako), anti-HER3 (1:30; C-17; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-pHER3 (1:30; 21D3; Cell Signaling, Beverly, MA, USA), anti-PTEN (1:100; 6H2.1, Dako), anti-TYMS (1:60; TYMS106, Thermo Fisher, Fremont, CA, USA), and anti-TYMP (1:60; PGF.44C; Thermo Fisher).

IHC staining was scored and confirmed by two pathologists who were blinded to the clinical information. Positive ER and PR expression were defined as nuclear staining in 10% or more of tumour cells. EGFR overexpression was classified as weak to moderate or strong membranous staining in more than 10% of tumour cells. HER2 membranous staining was scored on a scale of 0 to 3+ according to the HercepTest protocol (6). For tissue samples with a HER2 staining score of 2+, additional HER2 FISH testing was performed. HER2 status was considered positive when the IHC score was 3+ or the gene copy ratio of *HER2*/*CEP17* by FISH was 2.2 or higher. For HER3, pHER3 and PTEN, the intensity (0, none; 1, weak; 2, moderate; 3, strong) and extent (0%-100% of tumour cells) of IHC staining in both the cytoplasm and membrane were determined. For HER3 and pHER3, a staining intensity score of 2 or more was defined as positive. Cytoplasmic PTEN expression was scored semiquantitatively using the immunoreactive score (IRS) as described elsewhere (7). An IRS of 0-3 was defined as low PTEN expression. Vascular endothelium, which is known to express normal PTEN, was used as a positive internal control. For TYMS and TYMP, intensity (0-3) and extent of staining in both the cytoplasm and nucleus were scored. Low expression of TYMS and TYMP was defined as cytoplasmic or nuclear staining with an intensity score of 0 or 1, whereas high expression was defined as cytoplasmic or nuclear staining with an intensity score of 2 or 3.

Statistical analyses. Categorical variables were compared using Pearson's χ^2 test or Fisher's exact test, where appropriate. TTP and OS were defined as the time from treatment initiation to the time of disease progression and death from any cause, respectively. The Kaplan–Meier method was used to estimate the median TTP and OS. TTP and OS comparisons were performed using the log-rank test. Multivariate analyses of TTP and OS were conducted using the backward stepwise Cox proportional hazard regression model after adjusting for age, hormone receptor status, number of prior chemotherapies (<4 *vs.* ≥4), and prior exposure to capecitabine. All *p*-values were two-tailed, and $p<0.05$ was considered statistically significant.

Table I. *Baseline patient characteristics*.

Characteristic	Patients (N=35)
Median age (range), years	52 (32-70)
ECOG performance scale, N (%)	
0	1 (2.9)
1	34 (97.1)
2	0 (0.0)
Disease status at treatment, N (%)	
Initially metastatic	3 (8.6)
Relapse	32 (91.4)
Number of metastatic organs, N (%)	
1	5 (14.3)
2	19 (54.3)
≥3	11 (31.4)
CNS involvement, N (%)	15 (42.9)
No. of previous palliative chemotherapy, median (range)	3 (1-9)
Previous exposure to capecitabine, N (%)	14 (40.0)

ECOG: Eastern Cooperative Oncology Group; CNS: central nervous system.

Table II. *Treatment outcomes of lapatinib plus capecitabine according to immunohistochemical expression.*

		RR, N (%)	*p*-Value	TTP, months (95% CI)	*p*-Value	OS, months (95% CI)	*p*-Value
All patients		8/35 (22.9%)		5.8 (3.6-8.0)		13.6 (10.3-16.8)	
ER	–	7/29 (24.1%)	0.916	5.6 (3.1-8.1)	0.438	12.4 (7.8-17.1)	0.503
	+	1/6 (16.7%)		5.9 (0.0-11.9)		13.8 (3.2-24.3)	
PR	–	8/28 (28.6%)	0.240	5.6 (3.1-8.1)	0.744	12.4 (8.9-16.0)	0.838
	+	0/7 (0.0%)		5.9 (0.0-12.8)		13.8 (0.0-34.0)	
EGFR	–	6/23 (26.1%)	0.140	5.9 (3.2-8.5)	0.628	11.3 (6.8-15.7)	0.717
	+	0/5 (0.0%)		5.8 (5.4-6.2)		15.8 (11.0-20.6)	
HER2	2+	1/8 (12.5%)	0.647	3.1 (2.0-4.3)	0.212	5.5 (1.0-10.1)	0.003
	3+	7/27 (25.9%)		6.1 (5.2-7.0)		15.1 (11.8-18.4)	
HER3	–	1/8 (12.5%)	0.218	3.8 (0.0-9.7)	0.938	7.1 (5.0-9.2)	0.293
	+	5/20 (25.0%)		5.8 (4.8-6.8)		15.8 (12.6-19.0)	
pHER3	–	3/17 (17.6%)	0.579	5.8 (3.1-8.5)	0.549	11.3 (3.2-19.4)	0.648
	+	3/11 (27.3%)		6.3 (4.6-8.0)		15.1 (9.1-21.0)	
PTEN	–	1/11 (9.1%)	0.331	5.8 (2.6-9.0)	0.669	11.3 (3.3-19.2)	0.911
	+	5/17 (29.4%)		6.1 (2.7-9.5)		15.1 (9.1-21.1)	
TYMS	–	4/22 (18.2%)	0.384	5.8 (2.8-8.8)	0.874	13.8 (2.4-25.3)	0.241
	+	2/6 (33.3%)		5.6 (4.2-7.0)		10.9 (7.7-14.2)	
TYMP	–	5/20 (25.0%)	0.501	5.6 (1.7-9.5)	0.403	11.3 (5.8-16.7)	0.192
	+	1/9 (11.1%)		5.9 (3.8-7.9)		17.1 (7.3-26.9)	

RR: Response rate; TTP: time to progression; OS: overall survival; CI: confidence interval; ER: oestrogen receptor; PR: progesterone receptor; EGFR: epidermal growth factor receptor; HER: human epidermal growth factor receptor; pHER3: phosphorylated HER3; PTEN: phosphatase and tensin homologue; TYMS: thymidylate synthase; TYMP: thymidine phosphorylase.

Results

Patient characteristics and treatment outcomes. A total of 35 patients were enrolled in the study. The baseline characteristics of the patients are presented in Table I. At a median follow-up duration of 25.9 months (range=13.4-33.9 months), all 35 patients had discontinued treatment because of disease progression (34 patients) or consent withdrawal (one patient), and 32 patients (91.4%) had died. The response rate (RR) was 22.9%. The median TTP and OS were 5.8 months [95% confidence interval (CI)=3.6-8.0 months] and 13.6 months (95% CI=10.3-16.8 months), respectively.

IHC features associated with sensitivity to lapatinib-plus-capecitabine. Pre-treatment biopsy samples were used in 13 (37.1%) patients; for the other 22 patients, archival tumour tissues were used. All patients were HER2 2+ or 3+ by IHC and all eight patients with HER2 IHC 2+ were positive for HER2 FISH (median=6.94; range=3.84-12.11) (Table II). Patients with high HER2 expression (3+) (77.1%) had superior OS [15.1 months (95% CI=11.8-18.4 months) *vs*. 5.5 months (95% CI=1.0-10.1 months); p=0.003] and showed a tendency towards a higher RR (25.9% *vs*. 12.5%; p=0.647) and longer TTP [6.1 months (95% CI=5.27.0 months) *vs*. 3.1 months (95% CI, 2.0-4.3 months); p=0.212] than those with low HER2 expression (2+) (Figure 1). Treatment outcomes were not significantly associated with expression levels of ER, PR, EGFR, HER3, pHER3, PTEN, TYMS, or TYMP by IHC. High HER2 expression was the only parameter significantly associated with longer OS (hazard ratio=3.56, 95%CI=1.48-8.58; p=0.005).

IHC features associated with trastuzumab resistance. A total of 49 tumor tissues including 14 paired samples were categorized as trastuzumab-resistant (n=16) and trastuzumab-sensitive (n=33). Positivity for pHER3 (66.7% *vs*. 20.8%; p=0.007) and TYMS (40.0% *vs*. 8.0%; p=0.036) was significantly more frequent in trastuzumab-resistant tumours than in trastuzumab-sensitive tumours (Table III). Positivity for TYMS expression was more frequent in trastuzumab-resistant tumours, both in patients with (50.0% *vs*. 11.1%) and without (33.3% *vs*. 6.2%) prior exposure to capecitabine. PTEN showed a tendency towards being more frequently expressed in trastuzumab-resistant tissues (73.3% *vs*. 40.0%; p=0.055). The expression of ER, PR, EGFR, HER2, HER3, and TYMP was not significantly different between trastuzumab-resistant and trastuzumab-sensitive tumours.

In 14 patients with paired tumor samples, discordance rates were 35.7% for ER, 14.3% for PR, 21.4% for EGFR, 35.7% for HER2, 27.3% for HER3, 36.4% for pHER3, 41.7% for PTEN, 50.0% for TYMS, and 25.0% for TYMP. Notably, discordant expression of pHER3, TYMS, and PTEN was due to an elevated expression in most cases (100%,

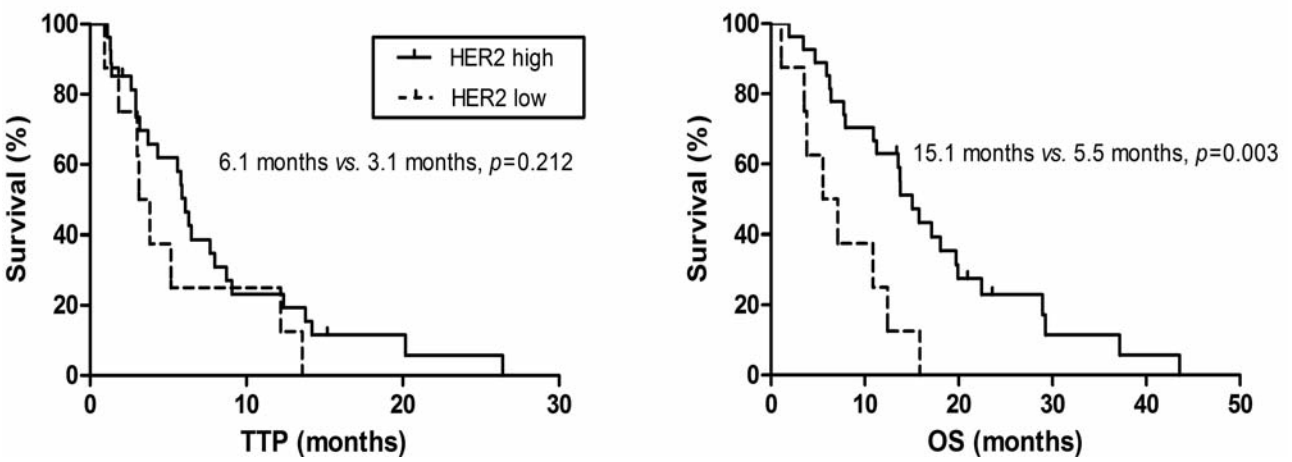

Figure 1. *Time-to-progression (TTP) and overall survival (OS) according to human epidermal growth factor receptor 2 (HER2) levels (p-values according to log-rank tests).*

pHER3 and PTEN; 66.7%, TYMS). An increase in pHER3 expression was associated with a superior RR (75.0% *vs.* 0.0%; p=0.024) and TTP (8.7 months *vs.* 1.8 months; p=0.040) and a tendency towards longer OS (10.9 months *vs.* 6.4 months; p=0.568) (Figure 2). However, changes in TYMS and PTEN expression were not associated with treatment outcome.

Discussion

In our study, high HER2 expression by IHC (3+) was associated with superior treatment outcomes in patients with HER2-positive MBC treated with lapatinib-plus-capecitabine. This is in line with previous biomarker analyses of a phase III study, which demonstrated that progression-free survival improvement with lapatinib plus capecitabine was not evident in patients with a HER2 IHC score of less than 3+, irrespective of FISH results (8). Our prior study, using novel quantitative HER2 assay, also indicated that a high HER2 level was predictive of a longer TTP and higher RR for lapatinib-plus-capecitabine treatment (9). Together, these data support that HER2-positive tumours resistant to the anti-HER2 drug trastuzumab are still dependent on the HER2 signalling pathway and the level of HER2 expression may provide additional information in identifying patients most likely to benefit from lapatinib plus capecitabine treatment.

Up-regulation of pHER3 and TYMS was associated with trastuzumab-resistant breast cancer in this study. Intriguingly, patients with an increased HER3 IHC score in matched pairs demonstrated a higher RR and longer survival times with lapatinib and capecitabine therapy. Our findings support the significance of HER3 signaling in the development of trastuzumab resistance, as well as in the mechanisms of action of lapatinib. Trastuzumab is effective against HER2 homodimers, but not against ligand-dependent HER2 heterodimerisation with other HER family members (EGFR and HER3), because it binds to a region of HER2 that is not involved in receptor dimerization (10). Therefore, signalling *via* other HER members, especially HER3, has been implicated as an important mechanism of trastuzumab resistance (10, 11). On the other hand, lapatinib can inhibit HER3 phosphorylation by HER2 tyrosine kinase and thereby abolish HER3 signaling (10, 11). In our patients

Table III. *Immunohistochemical features associated with trastuzumab resistance.*

	Trastuzumab-sensitive	Trastuzumab-resistant	*p*-Value
ER+	8/33 (24.2%)	3/16 (18.8%)	>0.999
PR+	8/33 (24.2%)	3/16 (18.8%)	>0.999
ER+/PR+	11/33 (33.3%)	3/16 (18.8%)	0.336
EGFR+	2/24 (7.7%)	4/16 (25.0%)	0.180
HER2			0.709
1+ or 2+	6/33 (18.2%)	4/16 (25.0%)	
3+	27/33 (81.8%)	12/16 (75.0%)	
HER3+	15/24 (62.5%)	13/15 (86.7%)	0.150
pHER3+	5/24 (20.8%)	10/15 (66.7%)	0.007
PTEN+	10/25 (40.0%)	11/15 (73.3%)	0.055
TYMS+	2/25 (8.0%)	6/15 (40.0%)	0.036
TYMP+	8/26 (30.8%)	5/15 (33.3%)	>0.999

ER: Estrogen receptor; PR: progesterone receptor; EGFR: epidermal growth factor receptor; HER: human epidermal growth factor receptor; pHER3: phosphorylated HER3; PTEN: phosphatase and tensin homologue; TYMS: thymidylate synthase; TYMP: thymidine phosphorylase.

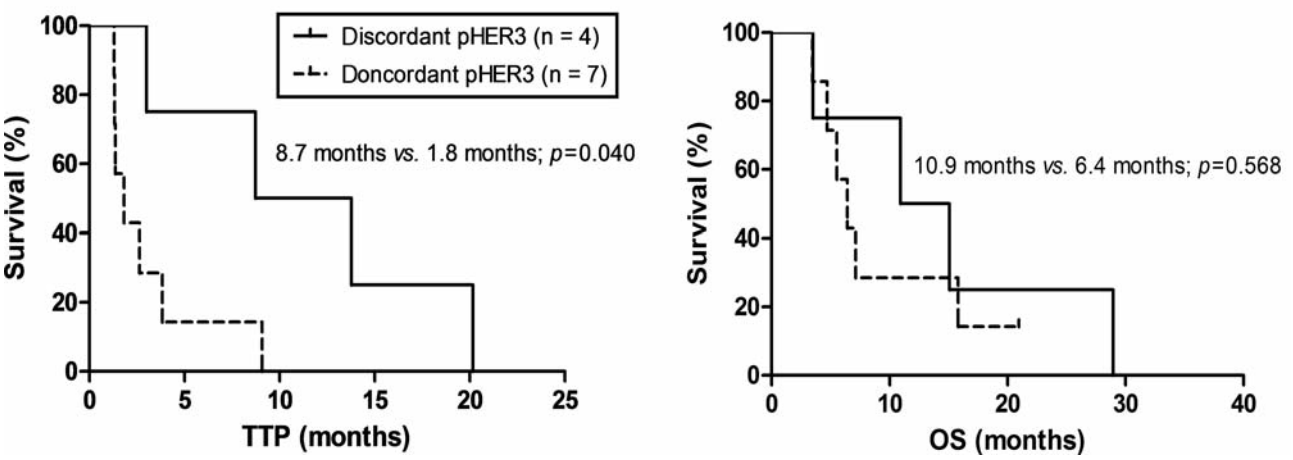

Figure 2. *Time-to-progression (TTP) and overall survival (OS) according to discordance in phosphorylated human epidermal growth factor receptor 3 (pHER3) expression in 14 patients with paired tumour samples (p-values are by log-rank tests). Discordant expression of pHER3 was due to an elevated expression in all 4 cases.*

with increased pHER3 expression, the favourable clinical outcome with lapatinib-plus-capecitabine suggests that lapatinib may effectively overcome trastuzumab resistance induced by HER2/HER3 signaling. In the present study, TYMS expression was up-regulated in trastuzumab-resistant tumors and was not affected by prior exposure to capecitabine. Discordant TYMS expression between primary and metastatic tumors has mainly been reported in colorectal cancer (12), and few reports have been documented in breast cancer (13). TYMS expression has been shown to be dependent on EGFR and HER2 signalling. Forced expression of EGFR and HER2 by transfection has been shown to activate the *TYMS* gene promoter, whereas inhibition of EGFR and HER2 has been shown to down-regulate *TYMS* promoter activity (14). Therefore, *TYMS* gene expression may be increased in trastuzumab-resistant tumors with activated HER2 and HER3 signaling. Although TYMS is one of known targets of capecitabine, the predictive role of high TYMS expression in treatment with capecitabine is largely unknown (15). More evidence is needed to determine if capecitabine is the optimal combination partner for lapatinib considering TYMS overexpression in trastuzumab-resistant tumours.

Herein, lapatinib and capecitabine treatment outcome was not significantly associated with expression of biomarkers by IHC other than HER2. A plausible explanation for this is that the IHC features associated with trastuzumab resistance change during the course of treatment and tumour progression. Expression of biomarkers by IHC changed considerably between paired trastuzumab-resistant and trastuzumab-sensitive tumour tissues, especially for pHER3, PTEN, and TYMS. Therefore, it might be inappropriate to correlate treatment outcome with biomarkers that vary over time.

The main limitations of this study include the small sample size, the limited number of biomarkers, the retrospective nature of the IHC testing, the single-arm study without a control group, and the combination treatment with capecitabine. Therefore, the results should be interpreted cautiously and warrant validation in prospectively designed studies with a larger sample size. However, in certain respects, the results are meaningful. Firstly, our data support the notion that HER2 levels by IHC could further guide proper selection for lapatinib-plus-capecitabine treatment in patients with HER2-positive breast cancer. Secondly, we identified some IHC features associated with the development of acquired resistance to trastuzumab in HER2-positive breast cancers. Increases in pHER3 and TYMS expression were particularly remarkable on this regard, and increased pHER3 expression was predictive for outcome of treatment with lapatinib-plus-capecitabine. Thirdly, our data show that the expression of biomarkers changes remarkably during tumor progression and trastuzumab treatment. Therefore, serial changes in biomarkers should be taken into account when interpreting the results and in the future design of biomarker studies.

In conclusion, the present study identified up-regulation of pHER3 and TYMS expression as IHC features associated with trastuzumab-resistant HER2-positive breast tumors. High HER2 levels by IHC and increased expression of pHER3 were predictive of favourable outcomes of lapatinib- plus-capecitabine treatment in patients with HER2-positive MBC.

Disclosures

None.

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