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# Role of HER3 expression and PTEN loss in patients with HER2-overexpressing metastatic breast cancer (MBC) who received taxane plus trastuzumab treatment

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**Background:** The aim of this study was to investigate the role of human epidermal growth factor receptor (HER3) and PTEN expression in patients with HER2-overexpressing metastatic breast cancer (MBC).

**Methods:** One hundred twenty-five MBC patients who were treated with taxane plus trastuzumab chemotherapy as first-line therapy were included in this analysis. Immunohistochemical (IHC) staining with HER3 and PTEN antibodies were conducted retrospectively.

**Results:** Patients who had negative HER3 staining (62.4%) had a better progression-free survival (PFS) than did those who had positive HER3 staining ( $P=0.001$ ; median PFS, 21 vs 11 months). Patients who had a PTEN score  $>20$  (78.1%) showed longer PFS than did those with a PTEN score  $\leq 20$  ( $P=0.006$ ; median PFS, 13 vs 9 months). Patients who had a PTEN score  $>20$  exhibited a longer overall survival (OS) than did those with a PTEN score  $\leq 20$  ( $P=0.005$ ; median OS, 48 vs 25 months). HER3 negativity and PTEN loss were identified as independent risk factors for PFS. PTEN loss was identified as an independent risk factor for OS.

**Conclusion:** HER3 and PTEN expressions may be predictive markers, and PTEN expression may be a predictive and prognostic biomarker for trastuzumab treatment in HER2-positive MBCs.

Trastuzumab, a monoclonal antibody against the human epidermal growth factor receptor 2 (HER2), has changed the treatment paradigm in patients with HER2-positive breast cancer. However, resistance to trastuzumab is an emerging problem and several new HER2-directed strategies have been developed to overcome this resistance (Arteaga *et al*, 2012; Perez and Spano, 2012). Among the several mechanisms underlying trastuzumab resistance, alteration of the phosphatidylinositol 3-kinase (PI3K)/AKT signalling pathway, which can be activated by HER2, took an early lead in the current translational research in this field (Fabi *et al*, 2010; Zhang and Yu, 2010; Mukohara, 2011). Trastuzumab has been suggested to inhibit PI3K/AKT survival signalling, either by downregulating

HER2 signalling or by increasing PTEN membrane localisation and phosphatase activity, leading to a decline in the activation of the PI3K/AKT pathway and inhibition of proliferation (Molina *et al*, 2001; Yakes *et al*, 2002; Nagata *et al*, 2004; Berns *et al*, 2007). In addition, activation of HER-related receptors, such as the HER3 receptor, has been suggested to increase PI3K/AKT signalling, thereby limiting trastuzumab efficacy in preclinical studies (Berns *et al*, 2007; Sergina *et al*, 2007). It has also been suggested that PTEN, which is a negative regulator of PI3K/AKT signalling, is involved in tumour sensitivity to trastuzumab. Thus, PTEN loss is negatively correlated with clinical outcomes in patients treated with trastuzumab (Berns *et al*, 2007). However, the results remain

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contradictory (Barbareschi *et al*, 2012; Perez and Spano, 2012) and the role of these molecules needs to be determined.

The epidermal growth factor receptor (EGFR) and HER2 classically couple with the Ras-Raf-MEK-mitogen-activated protein kinase (MAPK)-dependent pathway, whereas HER3 is a potent activator of PI3K/AKT (Mosesson and Yarden, 2004; Krause and Van Etten, 2005). It has been shown that the HER2-HER3 heterodimer constitutes the most mitogenic receptor complex within the HER family (Citri *et al*, 2003). It has also been suggested that this heterodimer is a potent combination in mammary cell line tumorigenesis (Holbro *et al*, 2003). Several studies have demonstrated that HER3 is frequently co-expressed with HER2 in breast cancer; hence, it is possible that the HER3 receptor has a part in HER2-mediated breast carcinogenesis (Bieche *et al*, 2003; Witton *et al*, 2003; Sassen *et al*, 2008). The prognostic value of HER3 expression in breast cancer has been poorly documented and the available data are not conclusive (Lemoine *et al*, 1992; Gasparini *et al*, 1994; Quinn *et al*, 1994; Travis *et al*, 1996). Although the overexpression of HER3 has been linked to HER2 positivity (Gasparini *et al*, 1994) and lymph node involvement (Bieche *et al*, 2003), a definitive relationship between HER3 and survival has not been established.

On the basis of this background information, we investigated the role of HER3 and PTEN expression in patients with HER2-overexpressing metastatic breast cancer (MBC).

## MATERIALS AND METHODS

**Patients.** Between 2005 and 2011, 125 patients who were at least 20 years of age and were being treated with taxane plus trastuzumab as first-line chemotherapy for histologically confirmed HER2-positive MBC were included in this analysis. HER2 positivity was defined as an intensity of 3+ by immunohistochemistry (IHC) or as a HER2/centromeric probe for chromosome 17 (CEP17) ratio >2.0 by fluorescent *in situ* hybridisation (Wolff *et al*, 2007). All patients were required to have an Eastern Cooperative Oncology Group (ECOG) performance status of 0–2 and have available paraffin tissue blocks from metastatic sites or breast tumours for HER3 and PTEN IHC. In addition, patients were eligible if they had received neoadjuvant or adjuvant chemotherapy, including taxanes, anthracyclines, or hormonal therapy, at least 12 months before the onset of taxane plus trastuzumab therapy. Exclusion criteria were previous chemotherapy for MBC, history of neoadjuvant or adjuvant chemotherapy, including trastuzumab or other HER2-directed agents. Thirty-eight patients received a tri-weekly course of paclitaxel plus trastuzumab chemotherapy, and the remaining 87 patients received a triweekly course of docetaxel plus trastuzumab chemotherapy. The triweekly paclitaxel plus trastuzumab regimen consists of the triweekly administration of paclitaxel (175 mg m<sup>-2</sup> in bovine serum albumin (BSA)) and trastuzumab at a loading dose of 8 mg kg<sup>-1</sup>, followed by 6 mg kg<sup>-1</sup> every 3 weeks. The triweekly docetaxel plus trastuzumab regimen consists of the administration of docetaxel (75 mg m<sup>-2</sup> in BSA) every 3 weeks, plus administration of trastuzumab at the same dose as that used in the triweekly paclitaxel plus trastuzumab regimen. This study was approved by the Samsung Medical Center Institutional Review Board.

**Acquisition of clinical data.** Data regarding patient demographics, pathological classification, treatment response, progression-free survival (PFS), overall survival (OS), and adverse events were obtained retrospectively by reviewing medical records. Treatment response was evaluated using a spiral computed tomography (CT) scan and the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 (Eisenhauer *et al*, 2009).

**HER3 and PTEN IHC and measurements.** Tissue samples were fixed in buffered 4% formalin and embedded in paraffin. A slide with a representative tumour was selected, and an area of tumour was circled on the slide.

The standard laboratory protocols were followed for IHC. Antigen retrieval was performed on deparaffinised whole sections (5 µm) by using preheated citrate buffer (98 °C; 40 min). Tissue sections were treated with Peroxidase Blocking Reagent (Dako, Carpinteria, CA, USA) and Background Sniper (Biocare, Concord, CA, USA) before manual IHC staining. Slides were incubated with monoclonal antibody against HER3 (mouse monoclonal; M7297, Dako; 1:100; 1 h at room temperature) and rabbit monoclonal antibody against PTEN (rabbit monoclonal; Cell Signalling, Boston, MA, USA; 1:100; overnight at room temperature). Sections were transferred to a Dako Autostainer Plus (Dako Reference No. S3800) and incubated in secondary antibody (Dako Envision plus Dual Link Horseradish Peroxidase Kit; Dako Reference No. K4061). The high-sensitivity diaminobenzidine (DAB) Chromogenic Substrate System (Betazoid DAB, Biocare) was used for colorimetric visualisation followed by counterstaining with hematoxylin. HER3 staining was categorised by intensity as 0, 1+, 2+, and 3+. Samples with no staining at all, or membrane staining in <10% of the tumour cells, result in a score of '0'. A faint or barely perceptible incomplete membrane staining in >10% of the tumour cells is scored '1+'. A weak to moderate staining of the entire membrane in >10% of the tumour cells is

Table 1. Baseline characteristics

Characteristics		Values (%)
Age	Median (range)	48.4 (29.9–74.5)
HER2 status (N, %)	IHC (3+)	93 (74)
	IHC (2+) and FISH (+)	32 (26)
Estrogen receptor status (N, %)	Positive	55 (44)
	Negative	70 (56)
Progesterone status (N, %)	Positive	40 (32)
	Negative	85 (68)
HER3 status (N, %)	Positive	34 (30)
	Negative	78 (70)
PTEN status (N, %)	Positive	92 (81)
	Negative (loss)	22 (19)
EGFR (N, %)	Positive	39 (48)
	Negative	42 (52)
Disease status	Relapsed	81 (64.8)
	Initially metastatic	44 (35.2)
Prior treatments (N, %)	Surgery	86 (68.8)
	Neoadjuvant chemotherapy	12 (9.6)
	Adjuvant chemotherapy	69 (55.2)
	Adjuvant radiation therapy	67 (53.6)
No. of metastatic sites	1	72 (57.6)
	2	34 (27.2)
	3	15 (12.0)
	≥4	4 (3.2)
Sites of metastasis (N, %)	Lymph node	46 (36.8)
	Lung and/or pleura	46 (36.8)
	Liver	30 (24.0)
	Brain	10 (8.0)
	Bone	43 (34.4)
	Others	10 (8.0)
ECOG performance status	0 or 1	110 (88)
	2	15 (12)
Treatment regimen	Triweekly paclitaxel + trastuzumab	38 (30)
	Triweekly docetaxel + trastuzumab	87 (70)

Abbreviations: ECOG = Eastern Cooperative Oncology Group; EGFR = epidermal growth factor receptor; HER = human epidermal growth factor receptor; IHC = immunohistochemistry.

scored '2+' and a strong staining of the entire membrane in > 30% of the tumour cells results a score of '3+'. PTEN staining was scored as proportion (0–100% of invasive cells) and intensity (1+ to 3+; weak, moderate, strong) in cytoplasmic staining because there is no validated standard definition for PTEN positivity or loss on the basis of our extensive literature review and personal discussions. The staining in normal elements and stroma was also used as a positive internal assay control, as applicable. The antibody used also produced slight nuclear staining that appeared to reflect in lesser degree the cytoplasmic expression and was of unknown significance.

Additional IHC staining for EGFR (Abcam, Cambridge, UK) was performed in 81 of the cases. Immunohistochemical staining of EGFR was interpreted as 'positive' or 'negative' according to the degree of membrane staining (cutoff value, 10%).

**Statistical analyses.** Progression-free survival was measured from the first day of administration of first-line palliative chemotherapy after recurrence to disease progression or death. Overall survival was measured from the day of administration of first-line chemotherapy to death or the final follow-up day. In univariate analysis, independent sample *t*-tests and  $\chi^2$  tests were used for continuous and categorical variables, respectively. The Kaplan–Meier product-limit method was used to estimate the PFS and OS. The survival rates were compared using the log-rank test. Multivariate analysis of the independent prognostic factors for survival was performed using the backward stepwise (likelihood-ratio statistics based on the conditional parameter estimate) method of the Cox proportional hazard regression model with a 95% confidence interval (CI). We also incorporated the newly proposed Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria for tumour marker publications into this study (McShane *et al*, 2005). As recommended by the REMARK criteria, we included a diagram to describe the descriptive analysis of patient samples performed in this study.

## RESULTS

**Patient characteristics.** The medical records of 125 MBC patients collected between 2005 and 2011 were analysed for evaluable HER3

and PTEN protein expression. The clinicopathological characteristics of the 125 HER2-positive MBC patients whose tumour blocks were available for HER3 and PTEN IHC are shown in Table 1. The median age of all patients was 48.4 years (range, 29.9–74.5 years). Fifty-five patients (26%) were positive for the estrogen receptor (ER) and 40 patients (32%) were positive for the progesterone receptor. Eighty-one patients (64.8%) had recurrent disease after curative treatment, whereas 44 patients (35.2%) were initially diagnosed with metastatic disease. Eighty-six patients (68.8%) underwent surgery. Eighty-one patients (64.8%) received neoadjuvant or adjuvant chemotherapy, and 67 patients (53.6%) received adjuvant radiotherapy. The sites of metastasis were as follows: lymph node in 46 patients (36.8%), lung and/or pleura in 46 patients (36.8%), liver in 30 patients (24%), brain in 10 patients (8%), and bone in 43 patients (34.4%). One hundred ten patients (88%) had an ECOG performance status of 0 or 1 at the baseline. Treatment regimens were as follows: 38 patients (30%) received triweekly paclitaxel plus trastuzumab and 87 patients (70%) received triweekly docetaxel plus trastuzumab. The patients' clinicopathologic characteristics listed by HER3 and PTEN status are shown in Table 2.

**HER3 and PTEN expression.** HER3 IHC was graded from 0 to 3+ in membranous staining as described in Materials and Method. As there is no validated standard definition of HER3 positivity and PTEN loss, based on our extensive literature review and personal discussion, we defined it here as follows: HER3 positivity was defined as a grade >0 (1+ to 3+) with at least 1+ membranous staining. HER3 positivity was shown in 27.2% of the patients (Figure 1A; Supplementary Figure 1). PTEN IHC was scored as the multiplication of intensity and proportion and ranged from 0 to 300 in cytoplasmic staining. PTEN positivity was defined as a score >20 with at least 1+ cytoplasmic staining. PTEN positivity was found in 78.1% of patients (Figure 1B). The proportion of patients with concomitant HER3 positivity and PTEN negativity (PTEN loss) was 10.9% of all patients.

Among 34 patients with HER3-positive disease, 16 (47%) patients were ER-positive and 8 of 25 (32%) patients with PTEN loss were ER-positive. In addition to these, 4 of 12 (33%) patients with concomitant HER3-positive disease and PTEN loss were ER-positive.

Table 2. Patients' characteristics according to HER3 and PTEN expression status

	HER3 +	HER3 –	P-value	PTEN +	PTEN loss	P-value
ER positivity	37.2%	47.1%	0.327	39.3%	32.0%	0.642
EGFR positivity	42.9%	49.1%	0.630	42.4%	75.0%	0.026
Median number of HER2-directed therapy after progression to taxane plus trastuzumab	1	1	0.999	1	2	0.425
Median number of lines of chemotherapy after progression excluding HER2-directed therapy	8	7	0.542	7	6	0.424
<b>Metastatic sites</b>						
Liver	5.9%	10.3%	0.721	7.9%	16.0%	0.254
Lung	20.6%	28.2%	0.397	24.7%	10.0%	0.792
Brain	21.0%	6.1%	0.042	14.6%	20.0%	0.540
Bone	26.2%	33.2%	0.423	30.2%	22.4%	0.422
Soft tissue	0%	3.8%	0.552	2.2%	4.0%	0.528
Skin	2.9%	5.1%	0.519	4.5%	4.0%	0.699
<b>Number of metastatic sites</b>						
1	42.0%	75.9%	0.032	89.9%	88.0%	0.724
≥2	68.0%	24.1%		10.1%	12.0%	
Abbreviations: EGFR = epidermal growth factor receptor; ER = estrogen receptor; HER = human epidermal growth factor receptor.						

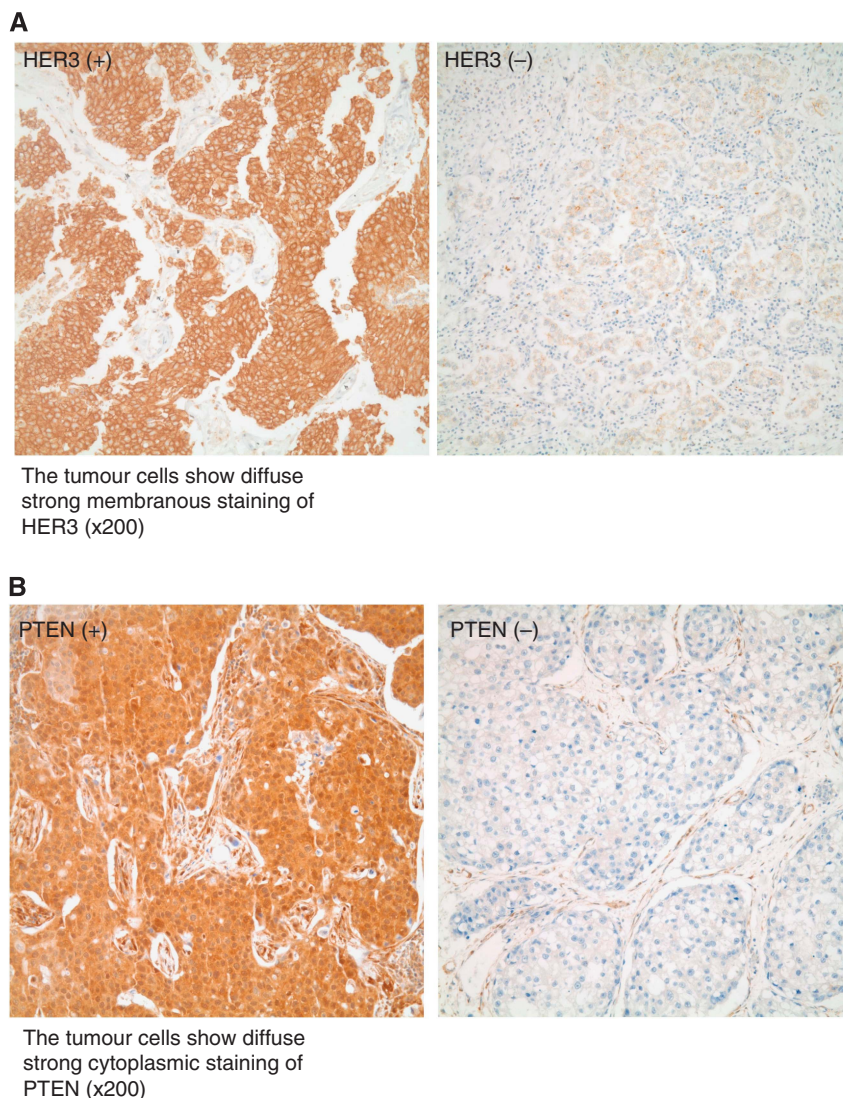
**EGFR expression.** Thirty-nine of 81 patients who were available for EGFR staining were EGFR-positive (48%). However, there has been no correlation between HER3 and EGFR positivity ( $P = 0.63$ ). However, the proportion of EGFR-positive patients were found more in PTEN-loss patients than in PTEN-positive patients (75% vs 42%,  $P = 0.026$ , Table 2).

**Relationship between HER2 expression and metastatic site with number.** The patients with HER3 positivity showed more CNS involvement at metastasis than those with HER3 negativity with statistical significance (21% vs 6%,  $P = 0.042$ ). Moreover, the proportion of patients with two or more metastatic sites was higher in HER3-positive than in HER3-negative patients (68.0% vs 24.1%,  $P = 0.032$ , Table 2).

**Prognostic significance of HER3 and PTEN expression including EGFR.** During a median follow-up of 36.0 months (range, 12.0–72.5 months), the median PFS and OS of all 125 patients were 12.6 months (95% CI, 10.0–15.2) and 44.3 months (95% CI, 36.3–52.6), respectively. The ER-positive patients had better outcomes than did the ER-negative patients in terms of PFS and OS, which are as follows: median PFS, 18.4 vs 10.7 months,  $P = 0.047$  (Figure 2A); median OS, 63.2 vs 43.4 months,  $P = 0.016$  (Figure 3A).

The patients who had negative HER3 staining showed a better PFS to taxane plus trastuzumab chemotherapy than did those who had positive HER3 staining ( $P = 0.001$ ; median PFS, 20.8 vs 10.5 months; Figure 2B). The patients who had a PTEN score  $> 20$  exhibited a longer PFS than did those with a PTEN score  $\leq 20$  ( $P = 0.006$ ; median PFS, 12.6 vs 8.5 months; Figure 2C). The patients who showed negative HER3 staining had a better OS to taxane plus trastuzumab chemotherapy than did those with positive HER3 staining; however, this result was not significant ( $P = 0.182$ ; median PFS, 'not reached' vs 43.5 months; Figure 3B). The patients who had a PTEN score  $> 20$  showed a longer OS than did those with a PTEN score  $\leq 20$  ( $P = 0.005$ ; median OS, 48.3 vs 24.9 months; Figure 3C). The patients who were EGFR-positive had a worse PFS and OS than those who were EGFR-negative (Figures 2D and 3D).

Estrogen receptor negativity, HER3 positivity, and PTEN loss were identified as independent risk factors for PFS in a multivariate Cox-regression model (HR = 1.67 (95% CI, 1.06–2.64),  $P = 0.027$  for ER negativity; HR = 2.43 (95% CI, 1.38–4.28),  $P = 0.002$  for HER3 negativity; and HR = 2.1 (95% CI, 1.2–3.7),  $P = 0.007$  for PTEN loss). However, ER negativity and PTEN loss (HR = 1.95 (95% CI, 1.01–3.78),  $P = 0.048$  for ER negativity;



**Figure 1. (A) HER3 staining.** Left; HER3-positive. The tumour cells show diffuse strong membranous staining of HER3 ( $\times 200$ ). Right; HER3-negative. **(B) PTEN staining.** Left; PTEN-positive. The tumour cells show diffuse strong cytoplasmic staining of PTEN ( $\times 200$ ). Right; PTEN-negative.

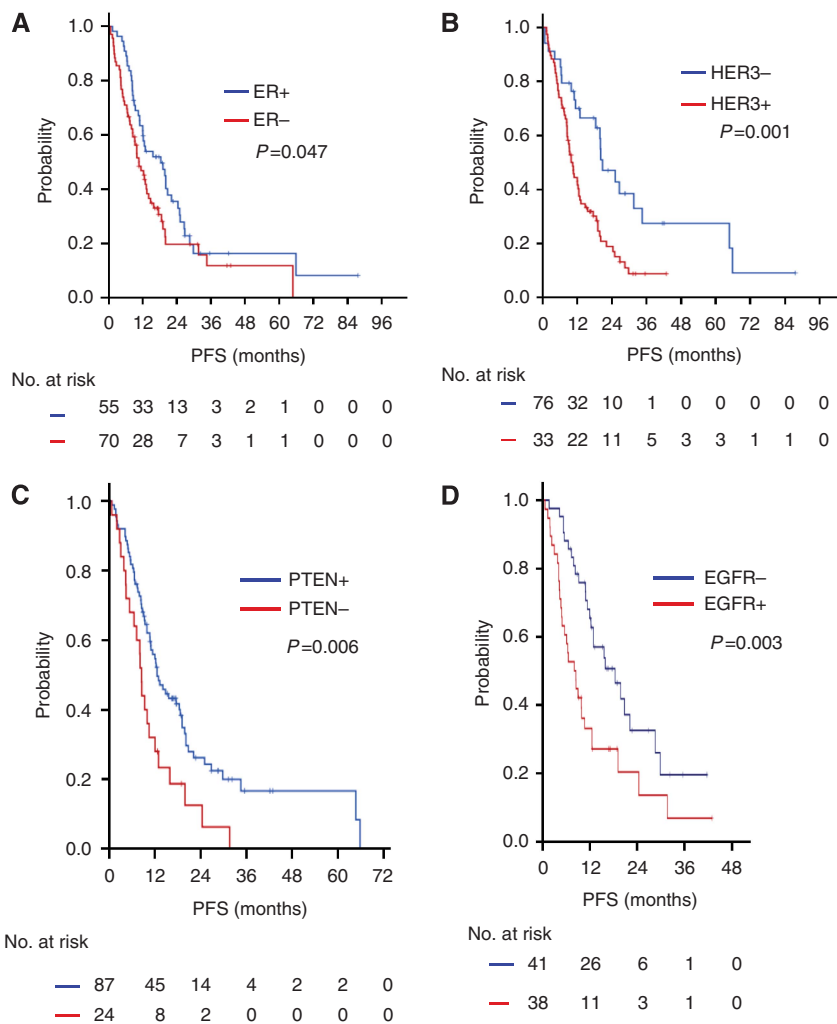


Figure 2. Kaplan–Meier PFS curves according to IHC. (A) PFS according to ER status. (B) PFS according to HER3 status. (C) PFS according to PTEN status. (D) PFS according to EGFR status.

HR = 1.56 (95% CI, 0.72–3.37),  $P=0.256$  for HER3 positivity; and HR = 3.2 (95% CI, 1.6–6.5),  $P=0.001$  for PTEN loss) were identified as an independent risk factor for OS (Table 3). In multivariate analysis, EGFR was not included because of small number of patients.

To evaluate HER3 positivity plus PTEN loss in HER2-overexpressing MBC patients, a log-rank test for Kaplan–Meier PFS and OS was performed. Patients with HER3 positivity plus PTEN loss had worse PFS and OS than did the remaining patients (median PFS, 6.5 vs 13.0 months,  $P=0.001$ ; median OS, 23.1 vs 48.3 months,  $P=0.002$ , Figures 4A and B).

## DISCUSSION

Despite the remarkable advancement in the treatment of HER2-overexpressing breast cancer provided by anti-HER2-directed therapy, especially trastuzumab, treatment resistance remains a main problem that needs to be solved. Among the few key mechanisms that underlie this phenomenon, signalling by other HER-family members, including HER3, and hyperactivation of the PI3K pathway associated with PTEN loss have been suggested to be the main causes of trastuzumab resistance (Yarden and Slivkowski, 2001; Nahta *et al*, 2006). PTEN loss in tumours leads to a lack in

negative regulation of PI3K signalling and aberrant activation of the transforming pathway. Thus, this regulation is particularly important in HER2-overexpressing breast cancer, which relies on HER2-activated PI3K for progression and survival (Yakes *et al*, 2002; Junttila *et al*, 2009). Furthermore, HER3 contains six PI3K-binding sites, which places the HER2–HER3 heterodimers among the most potent activators of the PI3K pathway (Holbro *et al*, 2003; Chan *et al*, 2005).

Surprisingly, one report showed no impact of PTEN loss in a large prospective adjuvant cohort (N9831 trial) (Perez *et al*, 2013). Those authors defined PTEN positivity as any staining of >0%. According to their results, the patients with HER2-positive early breast cancer with or without PTEN benefited from adjuvant trastuzumab. However, our data showed that PTEN loss resulted in worse outcomes in terms of PFS and OS in a cohort with metastatic cancer treated with taxane plus trastuzumab as the first-line treatment. These results were consistent with several PTEN-related results (Nagata *et al*, 2004; Fujita *et al*, 2006; Esteva *et al*, 2010; Razis *et al*, 2011). Although there are some plausible explanations for this discrepancy, metastatic retrospective cohort, IHC, and null PTEN with other aberrations in the PI3K pathway, more relevant clinical data are needed to evaluate this issue. In addition, our data suggest a role for the assessment of the concomitant expression of HER3 and PTEN loss using IHC in this clinical setting as a predictive biomarker of trastuzumab treatment. It is important to

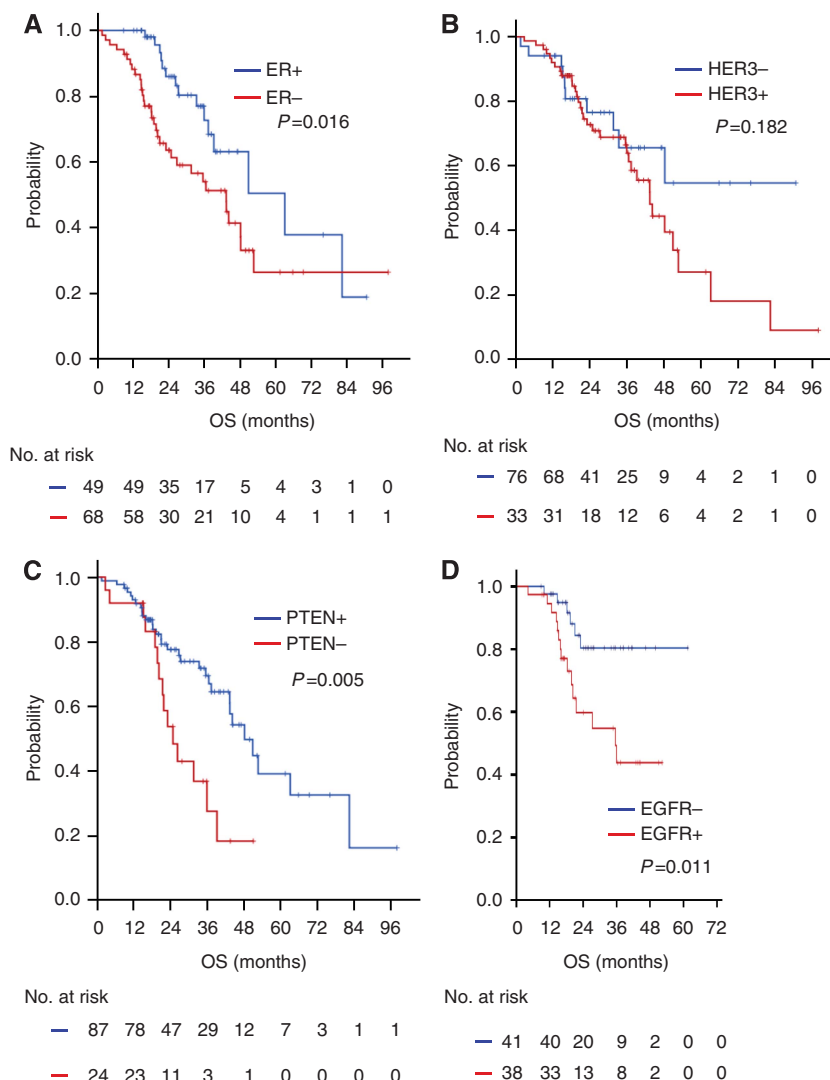


Figure 3. Kaplan–Meier OS curves according to IHC. (A) OS according to ER status. (B) OS according to HER3 status. (C) OS according to PTEN status. (D) OS according to EGFR status.

Table 3. Cox-regression multivariate analysis for PFS and OS

	PFS			OS		
	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
ER negativity	1.674	1.061–2.642	0.027	1.950	1.007–3.776	0.048
HER3 positivity	2.428	1.376–4.283	0.002	1.561	0.724–3.366	0.256
PTEN loss	2.124	1.224–3.686	0.007	3.190	1.576–6.458	0.001

Abbreviations: CI = confidence interval; ER = estrogen receptor; HER = human epidermal growth factor receptor; OS = overall survival; PFS = progression-free survival.

note that some patients who exhibited concomitant expression of HER3 and PTEN loss might have worse clinical outcomes to taxane plus trastuzumab treatment compared with other patients. Concomitant expression of HER3 and PTEN loss was present in 10.9% of all patients included in our cohort. This finding suggests that concomitant expression of HER3 and PTEN loss may explain the primary resistance to trastuzumab treatment in specific populations of cancer patients. Therefore, one of the strengths of

our study was the demonstration of the clinical implications of the concomitant expression of HER3 and PTEN loss. In contrast, it also had a major limitation—that is, retrospective design based on a small-sized cohort of metastatic patients.

Taken together, these data suggest that PTEN loss and HER3 positivity, as assessed using IHC, might be surrogate markers that can be used to predict outcomes to taxane plus trastuzumab treatment in patients with HER2-overexpressing

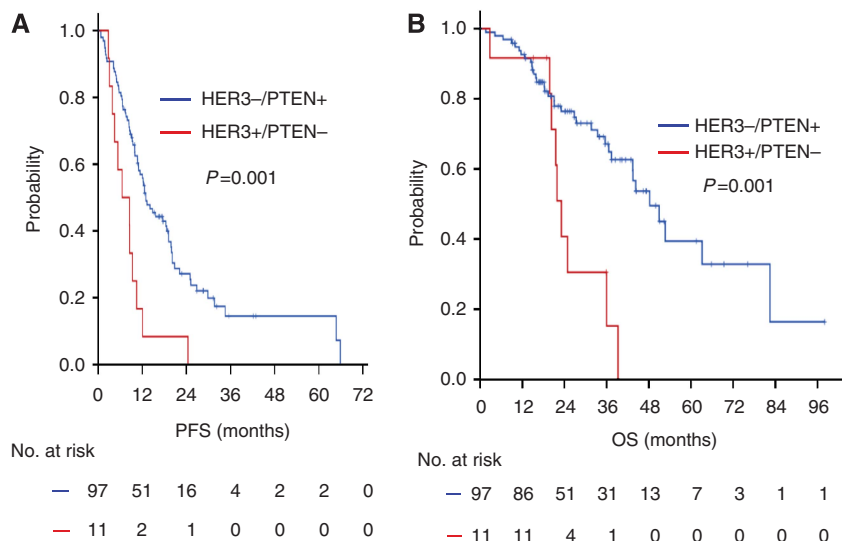


Figure 4. Kaplan-Meier PFS and OS curves. (A) PFS between the patients with HER3 - /PTEN + and those with HER3 + /PTEN loss. (B) OS between the patients with HER3 - /PTEN + and those with HER3 + /PTEN loss.

MBC. A validation study of a comprehensive set of genetic aberrations in the PI3K pathway is warranted.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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