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Clinical Significance of PTEN and p-Akt Co-Expression in HER2-Positive Metastatic Breast Cancer Patients Treated with Trastuzumab-Based Therapies

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Key Words
Metastatic breast cancer · Trastuzumab · p-Akt · Phosphatidylinositol 3′-kinase · Phosphatase and tensine homologue gene

Abstract
Objective: The phosphatase and tensine homologue gene (PTEN) plays a crucial role in proliferation and survival of cancer cells by antagonizing the function of phosphatidylinositol 3′-kinase (PI3K), which, in turn, results in decreased Akt activity. We investigated the clinical impact of the expression of PTEN, p-Akt and PI3K in HER2-positive metastatic breast cancer (MBC) patients treated with trastuzumab-based therapies. ***Methods:*** Seventy-three patients treated with trastuzumab-based therapies were included and followed prospectively. PTEN, p-Akt and PI3K expression was determined by immunohistochemistry. ***Results:*** PTEN, p-Akt and PI3K resulted positive in 48%, 71% and 46.5% of patients, respectively. A significant correlation between PTEN and p-Akt (kappa 0.22, p = 0.03) and p-Akt and PI3K (kappa 0.20, p = 0.05) was observed. PTEN-positive patients had a progression-free survival (PFS) longer than PTEN-negative ones (p = 0.06). When grouped together, patients co-expressing PTEN and p-Akt had a statistically significant longer PFS as compared to the rest of patients (p = 0.01). At the multivariate analysis, PTEN and p-Akt co-expression was an independent predictor of lower risk of progression (hazard ratio 0.53, p = 0.05). ***Conclusion:*** In HER2-positive MBC, basal co-expression of PTEN and p-Akt might identify those patients who are more likely to benefit from trastuzumab-based therapies.

Introduction

The monoclonal antibody trastuzumab (Herceptin®, Genentech, San Francisco, Calif., USA) was the first anti-HER2 agent which was registered for use in HER2-positive breast cancer [1]. Since its approval, trastuzumab has dramatically improved the outcome of HER2-positive disease, particularly in combination with chemotherapy [1–3]. As a result, trastuzumab-based therapies are currently the mainstay of treatment of HER2-positive breast cancer either in the (neo-)adjuvant or metastatic setting [4]. Despite these successes, clinical resistance to trastuzumab remains a significant problem. In HER2-positive metastatic breast cancer (MBC), 44–64% of patients show

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upfront resistance to single-agent therapy [5–7], whereas 12–22% of patients are primarily resistant to trastuzumab given in combination with various cytotoxics [2, 8, 9]. In addition to this, virtually all patients who initially benefit from trastuzumab-based chemotherapy will eventually develop progressive disease, usually within 1 year of treatment [10]. In this context, the identification of the cellular mechanisms responsible for trastuzumab resistance, based on the knowledge of the mechanisms underlying trastuzumab's antitumor activity, is of particular relevance. Despite uncertainty on how trastuzumab exerts its antitumor activity, preclinical and clinical evidence strongly suggests that inhibition of the phosphatidylinositol 3′-kinase (PI3K)/Akt pathway acts as the predominant mechanism among those proposed for trastuzumab's antitumor activity [11–13].

The phosphatase and tensine homologue gene (PTEN) is a tumor suppressor gene involved in the negative regulation of the PI3K/Akt pathway [14]. PTEN's inhibitory mechanisms is based on the prevention of phosphorylation of the serine/threonine kinase Akt to phospho-Akt (p-Akt) through opposition to the PI3K function. Functional loss of PTEN may derive from a number of circumstances such as protein downregulation, PTEN's gene deletions/mutations or promoter methylation [15–19]. However, loss of function due to protein downregulation has been shown to contribute significantly to resistance to treatment with trastuzumab both at a preclinical and clinical level [12, 20, 21]. On the other hand, preclinical evidence suggests that Akt is frequently phosphorylated in HER2-positive breast cancer [22–24], with rapid inactivation occurring right after trastuzumab exposure [12]. On this basis, we undertook a prospective evaluation of PTEN, p-Akt and PI3K expression by immunohistochemistry (IHC) in HER2-positive MBC patients receiving trastuzumab-based therapy and we studied the association of these proteins with clinical outcome both in terms of response rate and progression-free survival (PFS).

Materials and Methods

All data were collected at the Medical Oncology of the Regina Elena Cancer Institute in Rome, Italy. Assessment of HER2 status and IHC analysis of PTEN, p-Akt, and PI3K took place at the Department of Pathology of the Regina Elena Cancer Institute in Rome, Italy. The study was approved by local Ethics Committee and all patients gave written informed consent for marker analyses.

Patients Eligibility

Seventy-three consecutive HER2-positive MBC were included and followed prospectively. Patients were eligible if they were trastuzumab-naïve and had had histological diagnosis of breast cancer. Only patients eligible for trastuzumab in combination with chemotherapy were included in the study. No more than one prior line of chemotherapy for metastatic disease was allowed. Patients with asymptomatic and/or controlled brain metastases were eligible. HER2 was defined positive when tumors, using the polyclonal antibody A0485 (Dako, Milan, Italy), displayed an IHC score of 3+ or an IHC of score 2+ with gene amplification by fluorescence in situ hybridization (FISH) (PathVysion® assay kit, Vysis, Abbott, Ill., USA) or chromogenic in situ hybridization (CISH, Zymed, Histoline, Milan, Italy). Trastuzumab was given at 2 mg/kg weekly or 6 mg/kg every 3 weeks after a first infusion loading dose of 4 and 8 mg/kg, respectively. Disease progression following the most recent treatment prior to the initiation of trastuzumab-based therapy was documented in all patients. After initiation, trastuzumab was continued until disease progression unless withdrawal of the patient, unacceptable toxicity or deteriorated clinical conditions. In case of chemotherapy-related hematologic and/or nonhematologic toxicity, trastuzumab was maintained while dose modifications/omissions of the companion cytotoxic agents were performed appropriately.

Immunohistochemical Detection of PTEN, p-Akt, and PI3K

Immunohistochemical staining was carried out on tissue specimens obtained at the time of diagnosis. Two-nanometer-thick paraffin-embedded sections were stained with a streptavidin-enhanced immunoperoxidase technique (Supersensitive Multilink, Novocastra, Menarini Florence, Italy) in an automated autostainer (Bond Max, Menarini) using the following reagents: anti-PTEN (clone 28H6, Menarini), anti-p-Akt (Ser473 Cell Signaling, SIAL, Rome, Italy), anti-PI3K (clone 4, BD Transduction, SIAL). The pH 6 citrate buffer antigen retrieval protocol was applied for the three antibodies. Diaminobenzidine (Menarini) was used as chromogenic substrate. Sections were mounted in aqueous mounting medium (Glycergel, Dako).

Levels of PTEN expression were scored semiquantitatively based on staining intensity and distribution using the immunoreactive score (IRS) described elsewhere [12, 20, 21]. Briefly, IRS equals staining intensity × percentage of positive cells. Based on the IRS score, staining intensity was graded 0 (IRS 0–3), 1+ (IRS 4–6), 2+ (IRS 7–9) or 3+ (IRS 10–12). With regard to p-Akt, the scoring system previously described by Perez-Tenorio et al. [25] was used for protein evaluation. More in detail, tumors with a minimal or missing staining pattern were classified as negative (0). Tumors exhibiting a detectable, but faint immunostaining were scored as weakly positive (1+), whereas breast cancers showing a distinct and intense cytoplasmic immunostaining were scored as strongly positive (2+), regardless of the percentage of stained cells. For PTEN, a score of 0 or 1+ was considered negative. For p-Akt, a staining of 0 was regarded as negative. At present, there are no validated scoring systems for interpreting immunohistochemical staining for PI3K. Based on a quantitative evaluation, tumors were considered positive for PI3K when ≥50% of the neoplastic cells showed a distinct cytoplasmic staining.

Immunohistochemical staining was interpreted independently by two authors (A.D.B., M.M.) who were blinded to all patient

information. If discrepancies occurred, a consensus score was made by the two readers after discussion of the slide.

Statistical Methods

Descriptive statistics were used to summarize pertinent study information. The objective response rate was reported with its 95% CI. Disease response was categorized as progressive disease (PD), stable disease (SD) and complete or partial remission (CR and PR, respectively) according to RECIST [26]. The association between variables was tested by the Pearson χ^2 test or Fisher's exact test. Concordance between PTEN, p-Akt and PI3K was assessed using Cohen's k coefficient agreement. PI3K cut-off was calculated using the mean value of two different methods, ROC analysis and Classification and Regression Tree analysis (C and RT). Progression-free survival (PFS) and overall survival (OS) were calculated by the Kaplan-Meier method. The log-rank test was used to assess differences between subgroups. Significance was defined at the $p < 0.05$ level. PFS was calculated from the start of each trastuzumab-based therapy to the date of objective evidence of progressive disease or death of the patient in the absence of disease progression. Overall survival was recorded as the time elapsed from the date of first treatment to the date of death. If a patient had not progressed/died, PFS and OS were censored at the time of the last visit. Patients without an event were censored at the date of last follow-up. The hazard risk and the confidence limits were estimated for each variable using the Cox univariate model and adopting the most suitable prognostic category as referent group. Once verified the assumption of proportional risk a multivariate Cox proportional hazard model was also developed using stepwise regression (forward selection) with predictive variables which were significant in the univariate analyses. Enter limit and remove limit were $p = 0.10$ and $p = 0.15$, respectively. The SPSS (11.0) statistical program was used for analysis.

Table 1. Clinicopathological characteristics of the 73 HER2-positive MBC patients treated with trastuzumab-based therapies

Total number of patients (n = 73)	Frequency (%)
Median age (range)	47 (24–67)
Histotype	
Ductal carcinoma	69 (94.5)
Lobular carcinoma	4 (5.5)
Grading	
1–2	34 (46.5)
3	39 (53.5)
Prior exposure to anthracyclines	59 (81)
Anthracyclines in the (neo-)adjuvant setting	55 (75.5)
Anthracyclines in the metastatic setting	4 (5.5)
Prior exposure to a taxane*	20 (27.5)
Untreated for MBC	46 (63)
Predominant metastatic sites	
Liver	18 (24.5)
Lung	12 (16.5)
Bone tissue	13 (18)
Soft tissue	30 (41)
Trastuzumab-associated CT	
Taxane*	34 (46.5)
Vinorelbine	26 (35.5)
Capecitabine	13 (18)
Trastuzumab beyond progression	50 (68.5)
ER-positive	28 (38.5)
PgR-positive	26 (35.5)
PTEN-positive	35 (48)
p-Akt-positive	52 (71)
PI3K-positive	34 (46.5)

CT = Chemotherapy; ER = estrogen receptor; MBC = metastatic breast cancer; PgR = progesterone receptor.

* Paclitaxel or docetaxel.

Results

Patients Characteristics

Seventy-three patients were enrolled from a single institution from Feb. 2004 to Jan. 2007 (table 1). The median age was 47 years (range 24–67). Fourty-six patients (63%) received trastuzumab-based chemotherapy as first-line treatment for metastatic breast cancer. Trastuzumab was administered with a taxane (paclitaxel or docetaxel) in 34 (46.5%) patients, while 26 (35.5%) patients were treated with trastuzumab plus vinorelbine. In the remaining 13 (18%) patients, trastuzumab was administered with capecitabine.

PTEN, p-Akt, and PI3K Expression

Thirty-five (48%), 52 (71%) and 34 (46.5%) patients were found positive for PTEN, p-Akt and PI3K, respectively (table 1; fig. 1). No significant association was observed among PTEN, p-Akt or PI3K and any clinical or conventional biological characteristics (ER, PgR) (data not shown).

A statistically significant correlation was found between PTEN and p-Akt expression (kappa 0.22, $p = 0.03$). Similarly, p-Akt-positive expression was found to be significantly related to PI3K (kappa 0.20, $p = 0.05$). No statistically significant association was found between PTEN and PI3K positivity (kappa 0.15, $p = 0.21$).

Efficacy of Treatment

The median duration of trastuzumab-based therapies was 15.3 months (range 2.8–36.8). Thirty-seven patients responded to treatment (50.5%, 95% CI: 39.2–62.1) including 4 CR (5.5%) and 33 PR (45%). Stable disease was achieved in 19 patients (26%), while 17 patients (23.5%) experienced progressive disease. As of February 2008, 56

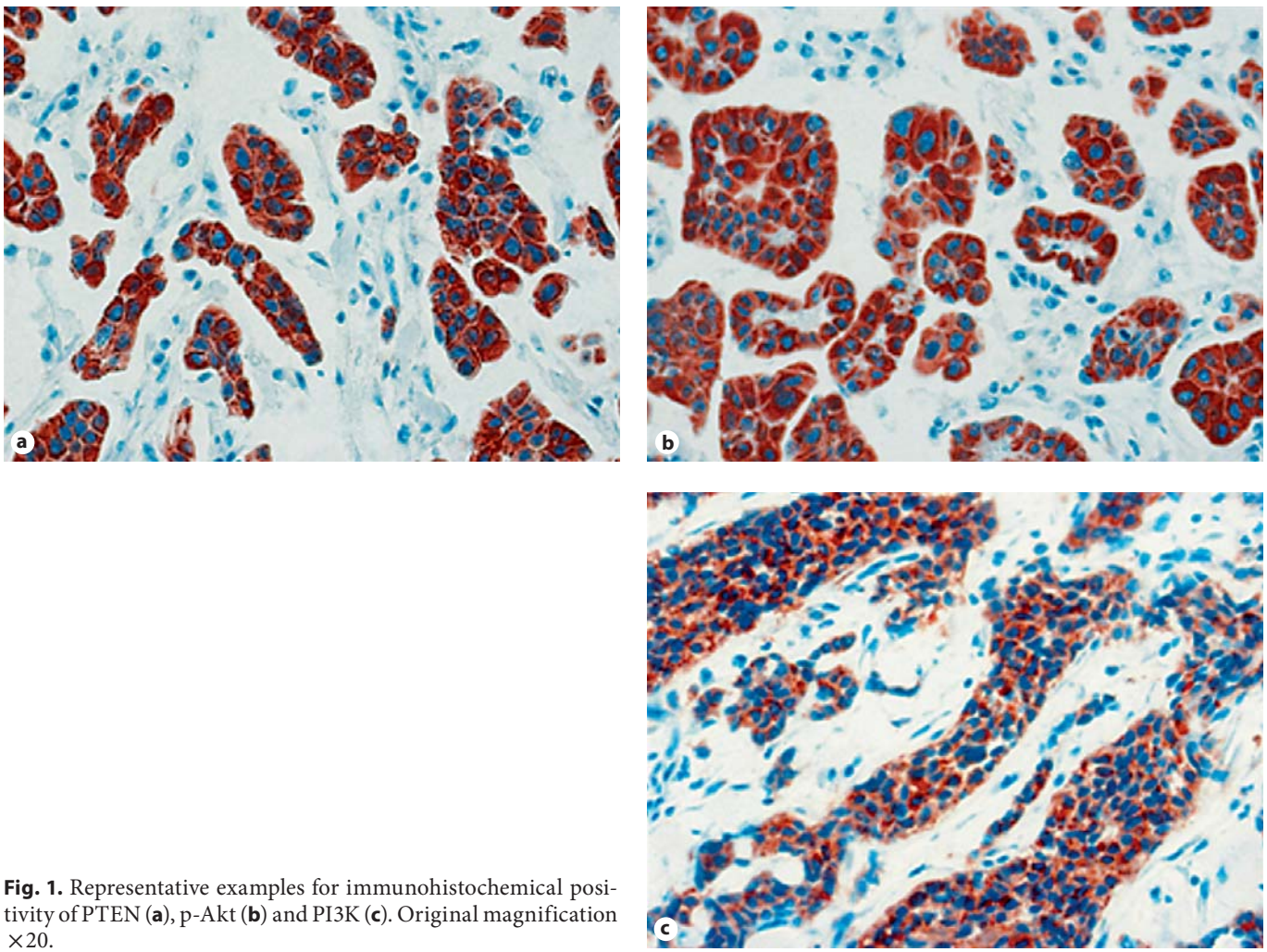


Fig. 1. Representative examples for immunohistochemical positivity of PTEN (**a**), p-Akt (**b**) and PI3K (**c**). Original magnification ×20.

patients had progressed and 36 had died. The median PFS was 8 months (95% CI: 8–12) and the 12- and 24-month PFS rates were 35.5 and 19.6%, respectively. The median OS was 30 months (95% CI: 13–47), and the 12- and 24-month OS rates were 56.3 and 49.1%, respectively.

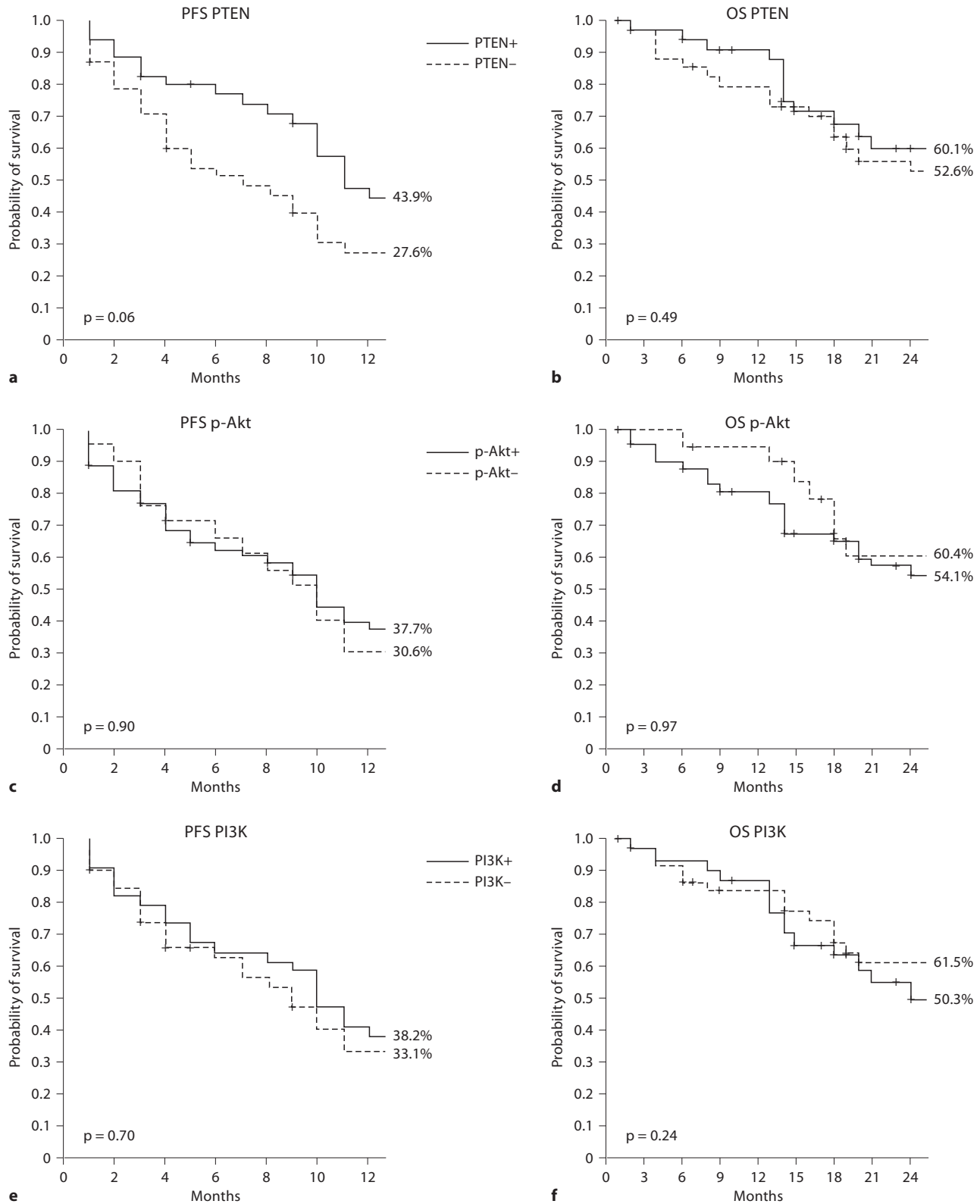
Clinical Outcome according to PTEN, p-Akt and PI3K Expression

No statistically significant association was observed between PTEN, p-Akt or PI3K and response to treatment ($p = 0.29$, $p = 0.22$ and $p = 0.56$, respectively, data not shown). As shown in figure 2, Kaplan-Meier curves showed a trend toward statistical significance with regard to PTEN positivity and longer PFS ($p = 0.06$). No other significant differences were observed with regard to PI3K or p-Akt status and PFS or overall survival (fig. 2).

Clinical Outcome according to Combined Expression of PTEN with p-Akt or PI3K

By combining PTEN with p-Akt or PI3K expression, patients were classified into 8 groups (table 2). No significant association with response was observed for any of the PTEN/p-Akt and PTEN/PI3K groups ($p = 0.11$ and $p = 0.64$, respectively, data not shown). By contrast, as summarized in table 2 and figure 3a, log-rank test showed that the 29 patients co-expressing PTEN and p-Akt displayed a significantly longer PFS (15 months) compared

Fig. 2. Kaplan-Meier estimates of progression-free survival (PFS) (**a**, **c**, **e**) and overall survival (**b**, **d**, **f**) for PTEN (**a**, **b**), p-Akt (**c**, **d**) and PI3K (**e**, **f**) protein expression. A trend toward statistical significance for longer PFS was noted only in PTEN-positive patients ($p = 0.06$). No other significant differences were observed.



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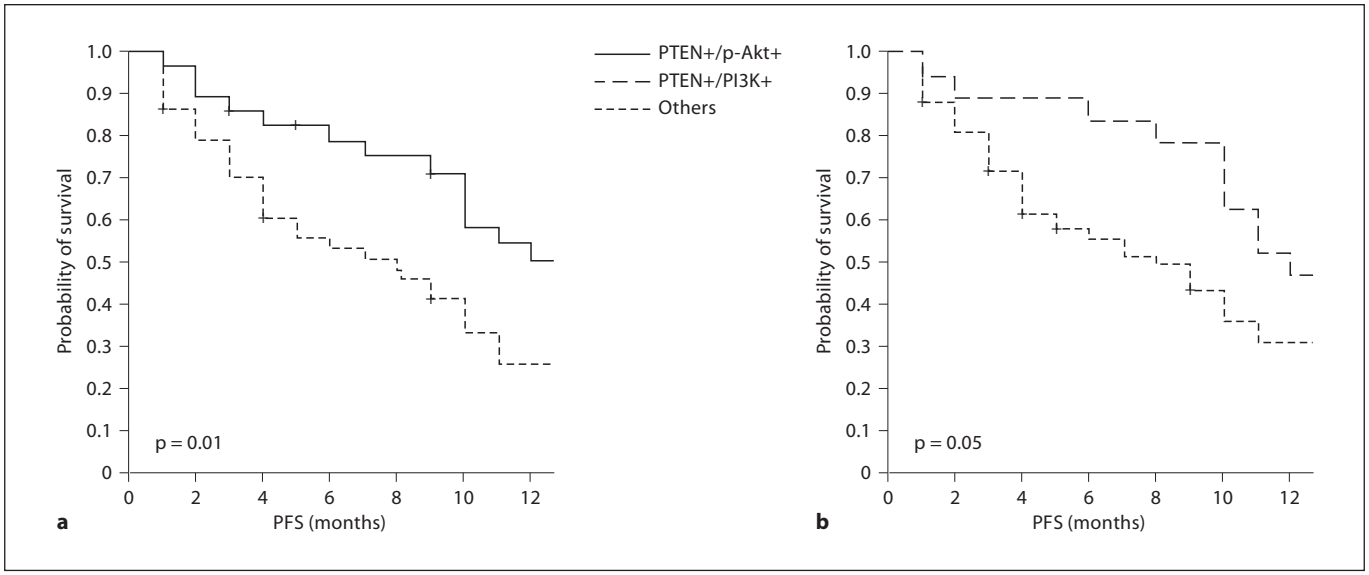

Fig. 3. a Kaplan-Meier estimates of PFS according to the combined expression of PTEN and p-Akt. PTEN+/p-Akt+ patients had a significantly longer PFS compared with others (15 months vs. 8 months, respectively, p = 0.01). **b** Kaplan-Meier estimates of PFS according to the combined expression of PTEN and PI3K. PTEN+/p-Akt+ patients showed a significantly longer PFS compared with the rest (12 months vs. 8 months, respectively, p = 0.05).

Table 2. PFS and OS according to the combined expression of PTEN with p-Akt or PI3K

Tumor phenotype	Median PFS* (95% CI)	1-year PFS (%)	p**	Median OS* (95% CI)	2-year OS (%)	p**
PTEN+/p-Akt+ (n = 29)	15 (7–23)	50.4		48 (14–82)	62.6	
PTEN+/p-Akt– (n = 6)	8 (2–14)	16.7	0.01§	18 (4–32)	50.0	0.30
PTEN–/p-Akt+ (n = 23)	5 (3–7)	21.5		20 (9–31)	43.3	
PTEN–/p-Akt– (n = 15)	10 (7–13)	36.7		38 (4–72)	65.3	
PTEN+/p-Akt+ (n = 29) vs. others (n = 44)	8 (4–12)	25.7	0.01§	27 (7–47)	52.3	0.21
PTEN+/PI3K+ (n = 19)	12 (4–20)	52.6		27 (14.40)	56.8	
PTEN+/PI3K– (n = 16)	11 (5–17)	42.4	0.10	48 (15–81)	64.5	0.57
PTEN–/PI3K+ (n = 15)	5 (3–7)	26.7		24 (8–40)	42.2	
PTEN–/PI3K– (n = 23)	8 (4–12)	27.7		38 (12–64)	59.4	
PTEN+/PI3K+ (n = 19) vs. others (n = 54)	8 (5–11)	31.5	0.05§	38 (14–62)	56.3	0.78

OS = Overall survival. * Months. ** log-rank test. § Statistically significant.

with the other 44 patients presenting alternative phenotypes (8 months) (p = 0.01). Similarly, a significantly longer PFS was observed for patients who were positive for both PTEN and PI3K (12 months) compared with the rest of patients (8 months) (p = 0.05) (table 2; fig. 3b). No statistically significant differences were found with regard to overall survival (table 2).

Univariate and Multivariate Analyses

Univariate analysis of PFS and OS included hormonal receptor status (positive vs. negative), no prior chemotherapy for MBC, presence of visceral metastases, response to treatment, type of trastuzumab-based chemotherapy (trastuzumab + taxanes vs. other treatments), positivity for PTEN, p-Akt or PI3K, and co-expression of PTEN and p-Akt or PTEN and PI3K. Only significant

Table 3. Factors emerging as significant predictors of lower risk of progression and death at the multivariate analysis

Factors	PFS		OS	
	HR (95% CI)	p*	HR (95% CI)	p*
Response to treatment	0.44 (0.23–0.83)	0.01	0.17 (0.07–0.42)	<0.0001
No prior therapy for MBC	0.26 (0.13–0.53)	<0.0001	0.33 (0.14–0.74)	0.002
PTEN+/p-Akt+	0.53 (0.29–0.99)	0.05	–	n.s.

MBC = Metastatic breast cancer; n.s. = not significant.
* Cox regression analysis using a forward stepwise procedure.

parameters were included in the multivariate Cox model. As illustrated in table 3, the multivariate analysis indicated that no prior chemotherapy for MBC and response to treatment were independent factors for lower risk of progression and death. Importantly, the multivariate analysis also showed that co-expression of PTEN and p-Akt was an independent predictor for lower risk of progression (hazard ratio 0.53, p = 0.05).

Discussion

Clinical resistance to trastuzumab-based therapies, either primary or acquired, is a relevant issue in HER2-positive metastatic breast cancer. The present study aimed at investigating prospectively the mechanisms involved in sensitivity to trastuzumab-based therapies through the measurement by IHC of three proteins that play a crucial role in the HER2-mediated signaling cascade, namely PTEN, p-Akt and PI3K.

Our series of HER2-positive MBC patients were treated with trastuzumab in combination with chemotherapy either as first- or second-line option. In this population, we found that 48% of patients showed PTEN positivity which is consistent with previous reports showing that PTEN expression in HER2-positive breast cancer is present in 35.5–78% of tumors [12, 20, 21, 27]. Nevertheless, we were unable to show a significant correlation between PTEN positivity and response to trastuzumab-based therapy, which appears in contrast with findings from two retrospective studies [12, 21]. However, it should be pointed out that the latter two studies were based on much smaller cohorts of patients (17 and 47 patients, respectively) than ours. In addition, the present study is to the best of our knowledge the first analysis prospectively evaluating PTEN in HER2-positive MBC patients treated with trastuzumab-based therapies. It is our opinion that response may not be the best outcome measure for addressing sensitivity to trastuzumab-based therapies according to PTEN expression. In fact, it can be speculated that the concomitant use of chemotherapy might mask the true impact of PTEN positivity on response to trastuzumab combined with chemotherapy. The biological explanation for this may rely on the fact that PTEN was found to be significantly associated with p-Akt (kappa 0.22, p = 0.03), thus suggesting that PTEN-positive patients are also likely to co-express p-Akt. Since constitutive and inducible Akt activity might be responsible for resistance to chemotherapy [22], PTEN-positive patients, who are theoretically more sensitive to trastuzumab, are at the same time potentially less sensitive to concomitant chemotherapy, owing to their increased likelihood of simultaneous expression of p-Akt. By contrast, PFS rather than response might best reflect the increased sensitivity of PTEN-positive patients to trastuzumab-based therapies [20]. The fact that we observed a longer PFS for PTEN-positive patients, with a Kaplan-Meier curve reaching a borderline significance (p = 0.06), is consistent with another report [20], and confirms that PTEN positivity could help select a population with increased sensitivity to trastuzumab-based therapies.

Importantly, we found that as much as 71% of patients were positive for p-Akt expression. This finding suggests that HER2-overexpressing breast cancer is in vivo highly dependent on Akt activation for proliferation and survival. Consistent with this finding, studies investigating p-Akt in human breast cancer specimens regardless of HER2 status have reported a statistically significant correlation between p-Akt expression and HER2 positivity [23, 28–30]. Moreover, preclinical data show that Akt is constitutively activated in HER2 overexpressing breast cancer cells [22, 23, 28], where Akt signaling has been

shown to act as major oncogenic determinant of the HER2-positive phenotype [31]. Notably, PTEN and p-Akt co-expression resulted to be significantly associated with longer PFS (table 2; fig. 3). Although it might be argued that the present study cannot discriminate precisely between the predictive and prognostic value of PTEN and p-Akt co-expression, preclinical evidence strongly suggests a predictive role for the simultaneous expression of these two markers. In fact, Akt activation does not preclude sensitivity to trastuzumab in HER2-overexpressing breast cancer cells, provided that functional PTEN is present [11]. As a result, it can be supposed that PTEN+/p-Akt+ breast cancers are those with an activated HER2 pathway, which, in turn, makes tumors with this phenotype more sensitive to trastuzumab-based therapies. On the other hand, patients with PTEN–/p-Akt+ tumors were reported to have the worse outcome in terms of PFS (table 2), which is in line with the data showing that aberrant Akt activation in the absence of PTEN's inhibitory effect renders HER2-overexpressing breast cancer primarily resistant to trastuzumab [11]. Importantly, in the multivariate analysis co-expression of PTEN and p-Akt emerged as an independent predictor of lower risk of progression (hazard ratio 0.53, p = 0.05; table 3).

As for PI3K, the finding that PTEN+/PI3K+ patients experienced a significantly higher PFS as compared to the rest of patients is tantalizing, although it should be noted that PI3K detection by IHC might not be actually measuring the functional protein. Nevertheless, dominant activating mutations of PI3K have recently emerged as predictors of a hyperactivated PI3K/Akt pathway [32], which makes detection of PI3K mutations as opposed to IHC detection a more appealing methodology for the identification of patients resistant to trastuzumab [20].

In conclusion, in our study basal co-expression of PTEN and p-Akt was able to identify a subset of HER2-positive MBC patients with increased likelihood of benefiting from trastuzumab-based therapies. This result might be important for the design of future correlative investigations in breast cancer patients. However, the selection of appropriate candidates for trastuzumab-based therapies requires further investigation. Evidently, additional biomarkers that still need prospective validation as well as a combination of multiple tests might allow us to define the population which is most sensitive to trastuzumab-based therapies.

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