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RESEARCH

p85 Protein Expression is Associated with Poor Survival in HER2-Positive Patients with Advanced Breast Cancer Treated with Trastuzumab

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Abstract To investigate the immunohistochemical expression of p85 in a cohort of trastuzumab-treated HER2-positive and HER2-negative metastatic breast cancer patients. The medical records of all patients with metastatic breast cancer treated with trastuzumab-based regimens between 1998 and 2010 were reviewed and clinical information was obtained. Formalin-fixed paraffin-embedded tumor tissue samples with adequate material were retrospectively collected

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from 183 patients. Samples were evaluated by immunohistochemistry for p85, estrogen receptors (ER), progesterone receptors (PgR), HER2, Ki67, PTEN and phosphorylated Akt (S473 and T308). HER2 status was studied by fluorescence in situ hybridization, as well. *PIK3CA* mutational status was also evaluated. Median follow-up for all patients was 72 months. Central re-evaluation for HER2 revealed only 111 HER2-positive cases, with the remaining 72 patients being HER2-negative. Median survival was longer in HER2-positive patients (50.7 months) compared to HER2-negative patients (36.6 months) both treated with trastuzumab, but this difference has not reached significance ($p=0.068$). In total, 62 % of the patients were found positive for p85, however the p85 protein was not found to be differentially expressed in HER2-positive versus HER2-negative cases. There were no significant associations between protein expression of p85 and any of the markers under study, or with time to progression. Positive p85 protein expression was however associated with poor survival in trastuzumab-treated HER2-positive patients. In our cohort of trastuzumab-treated HER2-positive breast cancer patients, positive p85 protein expression appears to be a prognostic factor of poor survival and, if validated, might have important implications in the treatment of such patients.

Keywords p85 · Prognostic factors · Breast carcinoma · Immunohistochemistry · HER2 status · Trastuzumab

Introduction

p85 is an enzyme that belongs to the class 1A phosphatidylinositol 3-kinases (PI3Ks), which are related to a wide range of oncoproteins and growth factor receptors [1]. More specifically PI3Ks have been found to interfere with many cellular processes, such as cell proliferation and survival, membrane trafficking, glucose transport, neurite outgrowth, membrane ruffling and superoxide production, as well as actin reorganization and chemotaxis [2, 3]. The above multiplicity of functions reflects the complexity of PI3Ks, which is highlighted by the isolation of three different classes featuring distinct signaling pathways [4].

In cancer, the most widely implicated class of PI3Ks is class1A, which are heterodimers comprising a p85 regulatory and a p110 catalytic subunit [5]. They are both activated by transmembrane tyrosine kinase receptors, such as EGFR, HER2 and IGF1-R [6, 7]. As a result there is phosphorylation of phosphatidylinositol biphosphate to phosphatidylinositol triphosphate and downstream activation of the PI3K/Akt pathway, which is simultaneously controlled by the inhibitory effect of the lipid phosphatase PTEN [8].

Recent research demonstrated that there is a direct positive regulation of PTEN by the p85 subunit of PI3K. p85 binds directly to and enhances PTEN lipid phosphatase activity. Moreover, p85 is crucial for the stabilization and localization of p110-PI3K enzyme activity, which generates the key signaling lipid phosphatidylinositol 3,4,5-triphosphate [9]. The latter is dephosphorylated by the PI3K-phosphatase PTEN. It is thereafter probable that p85 exerts both an enhancing and an inhibitory effect on the function of the PTEN/PI3K/Akt pathway. Nevertheless, in breast carcinoma, the regulatory function of p85 might be overwhelmed by the commonly observed activation of the PI3K/Akt pathway by factors, such as oncogenic *PIK3CA* mutations, which encode one of the p110 catalytic subunits of PI3K [10].

Taking under consideration the activation of p85 by HER2, as well as the multidirectional interference of p85 in the regulation of the PTEN/PI3K/Akt pathway, we sought to investigate, in the present study, the possible relation of the immunohistochemical expression of the p85 protein to the expression of PTEN and phosphorylated Akt, as well as to *PIK3CA* mutations in HER2-positive and HER2-negative metastatic breast carcinomas obtained from a cohort of patients treated with trastuzumab. The relation of p85 to parameters, such as time to progression and survival were also considered. The adverse prognostic effect of *PIK3CA* mutations and PTEN protein loss, in the same patient cohort, was previously reported [11].

Materials and Methods

The medical records of all patients with metastatic breast cancer treated with trastuzumab-based regimens between December 1998 and January 2010 were reviewed. Formalin-fixed paraffin-embedded (FFPE) tumor tissue samples were retrospectively collected from 246 breast cancer patients treated with trastuzumab-based regimens in the metastatic setting. Sixty-three cases were excluded for inadequate FFPE tumor tissue, thus decreasing the number of eligible/evaluable patients to 183. A REMARK diagram for the translational research studies is provided in Fig. 1. All carcinomas had initially been diagnosed as HER2-positive and thereafter all patients had been treated with transtuzumab. The translational research protocol has been approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Medicine (Protocol # 4283; January 14, 2008) under the general title “Investigation of major mechanisms of resistance to treatment with trastuzumab in patients with metastatic breast cancer”. All patients included in the study after 2005 provided written informed consent for the provision of biological material for future research studies, before receiving any treatment. Waiver of consent was obtained from the Bioethics Committee for patients included in the study before 2005.

All tumor samples were re-evaluated by immunohistochemistry for estrogen receptors (ER), progesterone receptors

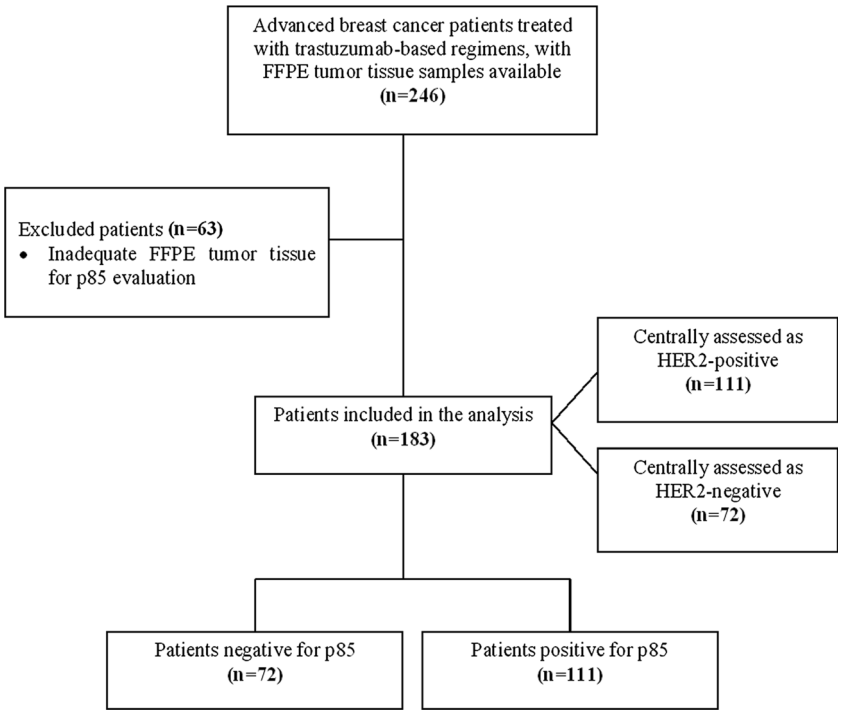

Fig. 1 REMARK diagram

(PgR), HER2 and the expression of the proliferation marker Ki67, while HER2 status was re-examined by fluorescence in situ hybridization (FISH). Moreover, for the purpose of the study, PTEN and phosphorylated Akt (473 and 308) were examined by immunohistochemistry, while single nucleotide polymorphism (SNP) genotyping was performed for the evaluation of *PIK3CA* mutations.

Tissue Microarrays (TMAs)

Fifteen TMA blocks were constructed out of the 183 cases using a manual tissue microarrayer (Beecher Instruments, Sun Prairie, WI). For the construction of TMA blocks, 2 core samples (1.5 mm in diameter) were obtained from representative regions of each of the tumor blocks. Each TMA block contained tissue cores from the original tumor tissue blocks, as well as cores from various neoplastic, non-neoplastic and reactive tissues, serving as assay controls. All markers were assessed by immunohistochemistry (IHC) or FISH in sections cut from the TMA blocks. Cases not represented, damaged or inadequate on the TMA sections were re-cut from the original blocks and these sections were used for protein and gene analysis.

Immunohistochemistry (IHC)

Three μm thick TMA sections or whole tissue sections were stained for ER (clone 6 F11, Leica Biosystems, Newcastle Upon Tyne, UK), PgR (clone 1A6, Leica Biosystems), Ki67 (clone MIB-1, Dako, Glostrup, Denmark), HER2 (A0482 polyclonal Ab, Dako), PTEN (clone 6H2.1, Dako, at dilution 1:200, for 30 min), pAkt Serine 473 ($pAkt^{473}$, clone 736E11, Cell Signaling Technology, Danvers, MA, at dilution 1:150, overnight at 4 °C) and pAkt Threonine 308 ($pAkt^{308}$, clone sc-16646-R, Santa Cruz Biotechnology, Santa Cruz, CA, at dilution 1:1000 overnight at 4 °C), using the Bond Max™ autostainer (Leica Microsystems, Wetzlar, Germany), as previously described in detail [12–15]. For the detection of the p85 protein the mouse monoclonal antibody raised against amino acids 333–430, mapping within the N-terminus SH2 domain of the 85 kDa subunit of PI3-kinase p85α of human origin was used (clone B-9, Santa Cruz Biotechnology) at dilution 1:100 for 60 minutes in an i6000™ autostainer (Biogenex, San Ramon, CA). All sections were stained in one run for each antibody and were evaluated by pathologists experienced in breast cancer and blinded as to the patient's clinical characteristics and survival data. Positive controls were used for all antibodies from known positive breast cancer cases, while negative controls were obtained by ommitment of the primary antibodies.

Interpretation of the IHC Results

Since p85 has not been found to be expressed in normal breast epithelium, any nuclear signal was considered as positive. ER and PgR immunostaining was scored using the Histoscore method. Tumors were classified as ER- or PgR positive if staining was present in 1 % or more of tumor nuclei [16]. HER2 protein expression was scored according to the recent guideline recommendations (scores from 0 to 3+) [17]. PTEN

protein expression (cytoplasmic, nuclear or both) was evaluated according to a staining intensity scale from 0 (negative, no staining) to 3 (intense staining). Tumors with PTEN scores of 0 or 1 were considered as having PTEN loss [11]. For Ki67, the expression was defined as low (<14 %) or high (≥14 %) based on the percentage of stained versus unstained tumor cell nuclei [18]. For the evaluation of $pAkt^{473}$ and $pAkt^{308}$, a Histo score based on the percentage of stained cells and the intensity of staining was calculated. A tumor was considered positive for $pAkt^{308}$ when >10 % of tumor cells were visualized with an intensity of 2 or 3 [14], while for $pAkt^{473}$ a cut-off point of >1 % of stained tumor cells was defined [15].

Fluorescence in Situ Hybridization (FISH)

TMA sections or whole tissue sections (5 μm thick) were used for FISH analysis using the ZytoLight® SPEC HER2/TOP2A/CEN17 triple color probe kit (ZytoVision, Bremerhaven, Germany). For all probes, sequential (5 planes at 1.0 μm) digital images were captured using the Plan Apo VC×100/1.40 objective (Nikon, Kanagawa, Japan), using specific filters for each probe. The resulting images were reconstructed using specially developed software for cytogenetics (XCyto-Gen, ALPHELYS, Plaisir, France). Four carcinoma cell lines (MDA-MB-231, MDA-MB-175, MDA-MB-453, and SK-BR-3) from the Oracle HER2 Control Slide (Leica) with a known *HER2* gene status were also used as a control of the FISH assays and analyzed for *HER2* genomic status.

FISH Evaluation

For the evaluation of *HER2* gene status, 20 non-overlapping nuclei from the invasive part of the tumor were selected randomly, according to morphological criteria using DAPI staining, and scored. The *HER2* gene was considered amplified when the ratio of the respective gene probe/centromere probe was >2.2 [17] and deleted when the ratio was ≤0.75. In cases at or near the cut-off (1.8-2.2 for amplifications and 0.6-0.9 for deletions), additional 20 to 40 nuclei were counted and the ratio was recalculated. In cases with a borderline ratio from 60 nuclei, an additional FISH assay was performed in a whole section, as previously described in detail [12].

Targeted PIK3CA Mutation Detection

DNA was extracted from 152 FFPE whole tissue sections or microdissected tissue fragments containing >70 % tumor cells, using a fully automated isolation method based on silica-coated magnetic beads (Versant Tissue Preparation Reagents, Siemens Healthcare Diagnostics, Tarrytown, NY). Targeted mutation testing for *PIK3CA* E542K and E545K (exon 9) and H1047R (exon 20) was accomplished with custom Taqman-MGB-SNP genotyping assays (duplex qRT-PCR for the detection of control DNA and mutant target in the same reaction) in an ABI7500 sequence detection system equipped with the SDS v1.4 software (Applied Biosystems/Life technologies, Paisley, UK) opting for Allelic Discrimination, as previously described [11].

Statistical Analysis

Follow-up information for all patients was updated in July 2012. All examined biomarkers are presented as frequency and corresponding percentages, while associations between them were examined using the chi-square test or Fisher's exact test where appropriate. Time to progression (TTP) was defined as the time from trastuzumab initiation in the 1st line treatment (with or without concurrent chemo/hormonal therapy) to the date of documented disease progression. Survival was also measured from the initiation of the trastuzumab treatment, in the patients receiving trastuzumab as a 1st line treatment, to the date of death. Patients were censored at the date of the last follow-up contact. Probabilities for TTP and survival were estimated by the Kaplan-Meier method. For the univariate and multivariate analyses, Cox proportional hazards models were used. Interaction tests of p85 with HER2 status in terms of TTP and survival were also examined.

In multivariate Cox regression analysis, model choice was performed using backward selection criteria with $p<0.10$, including in the initial step clinical parameters such as: age, menopausal status (post vs. pre), performance status (1–2 vs. 0), receptor status (ER/PgR) (positive vs. negative), Ki67 expression (high vs. low), number of metastatic sites (≥3 vs. 1–2), as well as the examined markers: HER2 status (positive vs. negative), p85 (positive vs. negative), *PIK3CA* (mutated vs. wild type), PTEN (no loss vs. loss), $pAkt^{473}$ (positive vs. negative) and $pAkt^{308}$ (positive vs. negative).

Results of this study are presented according to reporting recommendations for tumor marker prognostic studies [19]. All tests are two-sided at the alpha 5 % level of significance. No adjustment for multiple comparisons was performed. Analyses were conducted using the SPSS (version 15.0, IBM Corporation, Armonk, NY) and SAS (version 9.3, SAS Institute Inc., Cary, NC) software.

Results

Among the 246 registered patients with advanced breast cancer treated with trastuzumab, 183 patients were eligible and evaluable for p85 (Fig. 1). In total, 61 % of these patients were found positive for p85. Central re-evaluation for HER2 revealed that only 111 patients (61 %) were found to have centrally assessed *HER2* gene amplification by FISH and/or 3+ HER2 protein over expression by IHC (Fig. 1). Therefore,

a group of 72 HER2-negative patients was isolated. It is of note that all 183 patients were considered to be HER2-positive when assessed with IHC (and FISH in some cases) at the local laboratories and had therefore been treated with trastuzumab. Selected patient and tumor characteristics according to HER2 status are presented in Table 1.

Trastuzumab was given as 1st line treatment in 156 patients (85 %), while for the rest of the patients trastuzumab was initiated later in the course of metastatic disease. Most of the patients received trastuzumab in combination with chemotherapy (176 patients, 96 %), while the rest (7 patients, 4 %) received trastuzumab as monotherapy. Taxane based chemotherapy in addition to trastuzumab was the most frequent combination (123 patients, 67 %).

Association between the Examined Markers

The number of tumors that has been studied for each marker, as well as the expression of these markers in the present study population is shown in Table 2. Due to the detachment of core sections and the absence of adequate material for IHC evaluation in the respective whole sections, the final number of cases evaluated for each antibody varied.

p85 was found to be positive in 114 (62 %) of the patients. The association of p85 protein expression with all examined markers is shown in Table 3. The results did not reveal any statistically significant association between the expression of p85 and any of the markers under study nor with *PIK3CA* status. p85 was not found to be differentially expressed in HER2-positive vs HER2-negative cases. Moreover, no association was found between the expression of p85 and the grade of the carcinomas (p=0.87) (data not shown).

Effect of p85 on the Outcome of Advanced Breast Cancer Patients Treated with Trastuzumab

Median follow-up for all patients was 72 months (range, 0.5-110.1). Totally, 129 patients demonstrated tumor progression and 103 died among the 156 patients treated with trastuzumab in the 1st line setting. Median survival was longer in HER2-positive patients treated with trastuzumab (median survival 50.7 months, 95 % Confidence Interval [CI] 34.9-66.4) compared to HER2-negative patients (median survival 36.6 months, 95 % CI 32.0-41.3) but this difference did not reach significance (Wald's p=0.068). TTP was 16 months (95 % CI 10.8-21.6) for HER2-positive patients treated with 1st line trastuzumab, as compared to 10.2 months (95 % CI 7.7-12.8) for HER2-negative patients. This difference was also not statistically significant (p=0.095).

In univariate analysis, p85 was not associated with either survival (Hazard ratio [HR]=1.23, 95 % CI 0.82-1.83, Wald's p=0.32) or TTP (HR=0.89, 95 % CI 0.63-1.27, p=0.53). However, a statistically significant interaction between p85

Table 1 Selected patient and tumor characteristics (at trastuzumab initiation) according to HER2 status (HER2-positive: *HER2* amplified and/or HER2 IHC +3)

	HER2 status	
N=183	Negative *N*=72	Positive *N*=111
Age (years)		
Median (range)	58 (32–76)	55 (28–95)
	N (%)	N (%)
Menopausal status		
Premenopausal	23 (31.9)	34 (30.6)
Postmenopausal	49 (68.1)	77 (69.4)
Performance status		
0	46 (63.9)	78 (70.3)
1	14 (19.4)	21 (18.9)
2	6 (8.3)	5 (4.5)
Unknown	6 (8.3)	7 (6.3)
History of adjuvant CT	46 (63.9)	62 (55.9)
Anthracycline containing	24 (33.3)	49 (44.1)
Taxane containing	15 (20.8)	30 (27.0)
CMF-like	27 (37.5)	36 (32.4)
History of adjuvant HT	36 (50.0)	50 (45.0)
History of adjuvant RT	27 (37.5)	46 (41.4)
Histological grade (initial diagnosis)		
1	2 (2.8)	4 (3.6)
2	26 (36.1)	40 (36.0)
3	38 (52.8)	61 (55.0)
Unknown	6 (8.3)	6 (5.4)
Histological type (initial diagnosis)		
Invasive ductal	56 (77.8)	87 (78.4)
Invasive lobular	4 (5.6)	4 (3.6)
Mixed	5 (6.9)	5 (4.5)
Other	4 (5.6)	8 (7.2)
Unknown	3 (4.1)	7 (6.3)
Sites of metastases		
Locoregional	21 (29.2)	32 (28.8)
Only locoregional	5 (6.9)	6 (5.4)
Distant	62 (86.1)	95 (85.6)
Only distant	46 (63.9)	69 (62.2)
Bones	31 (43.1)	44 (39.6)
Visceral	46 (63.9)	79 (71.2)
Number of metastatic sites		
1	23 (31.9)	43 (38.7)
2	25 (34.7)	33 (29.7)
≥3	19 (26.4)	30 (28.3)
Unknown	5 (6.9)	5 (4.5)
Number of trastuzumab treatments		
1	32 (44.4)	42 (37.8)
2	18 (25.0)	30 (27.0)
3	9 (12.5)	19 (17.1)
≥4	13 (18.1)	20 (18.0)

CMF, Cyclophosphamide, methotrexate and 5-fluorouracil; *CT*, chemotherapy; *HT*, hormonal therapy; *N*, Number; *RT*, radiotherapy

Table 2 Distribution of the evaluated biological markers in the total cohort

		N %
ER (IHC) *n*=182	Negative	55 (30.2)
	Positive	127 (69.8)
PgR (IHC) *n*=182	Negative	91 (50.0)
	Positive	91 (50.0)
Ki67 (IHC) *n*=174	Low (<14 %)	12 (6.9)
	High (≥14 %)	162 (93.1)
PIK3CA n=152	WT	121 (79.6)
	Mutated	31 (20.4)
PTEN (IHC) *n*=175	Loss (intensity 0–1)	97 (55.4)
	No loss (intensity 2–3)	78 (44.6)
p85 (IHC) *n*=183	Negative	72 (39.3)
	Positive (any expression)	111 (60.7)
$pAkt^{473}$ (IHC) *n*=174	Negative	37 (21.3)
	Positive (>1 %)	137 (78.7)
$pAkt^{308}$ (IHC) *n*=175	Negative	60 (34.3)
	Positive (>10 % with 2, 3 intensity)	115 (65.7)

N, Number; *WT*, Wild-type

and HER2 status was found in terms of survival (Wald's p=0.034). Specifically, in HER2-positive patients a positive p85 expression was related to poor survival (HR=1.76, 95 % CI 1.03-3.01, Wald's p=0.039) compared to p85-negative tumors, while in HER2-negative cases no significant difference was found in terms of survival between cases featuring p85 positivity or no expression (HR=0.72, 95 % CI 0.38-1.35, Wald's p=0.30). In terms of time to progression (TTP) the interaction did not reach statistical significance, but a clear trend was detected (Wald's p=0.076). Specifically, in HER2-positive patients, there was no significant association between p85 and TTP (HR=1.12, 95 % CI 0.72-1.76, Wald's p=0.62), while in HER2-negative cases positive p85 tumors were marginally associated with longer TTP (HR=0.58, 95 % CI 0.33-1.03, p=0.062) compared to negative p85 tumors. Kaplan-Meier curves for survival and TTP, according to p85 protein expression in HER2-positive and HER2-negative patients, are shown in Fig. 2.

In multivariate Cox regression analysis (Fig. 3), the interaction of p85 with HER2 status in terms of survival remained significant. In HER2-positive patients, tumors with positive expression of p85 were associated with increased risk for death (HR=2.31, 95 % CI 1.31-4.09, Wald's p=0.004) compared to negative p85 tumors (Fig. 3a). Among the clinicopathological factors, only performance status remained in the final model with PS 1–2 patients having increased risk for death (HR=2.38, 95 % CI 1.54-3.67, p<0.001) compared to PS 0 patients. In terms of TTP, none of the parameters included in the final model reached statistical significance (Fig. 3b).

Table 3 Association of p85 with the rest of the examined markers

		p85				
		Negative		Positive		Fisher's exact *p*-value
		N	%	N	%	
ER	Negative	25	35.2	30	27.1	0.51
	Positive	46	64.8	81	72.9	
PgR	Negative	36	50.7	55	49.5	0.99
	Positive	35	49.3	56	50.5	
HER2	Negative	28	38.8	44	39.6	0.76
	Positive	44	61.2	67	60.4	
Ki67	Low	5	7.6	7	6.5	0.76
	High	61	92.4	101	93.5	
PIK3CA	WT	51	86.4	70	75.3	0.15
	Mutated	8	13.6	23	24.7	
PTEN	Loss	42	60.0	55	52.4	0.44
	No loss	28	40.0	50	47.6	
$pAkt^{473}$	Negative	19	27.1	18	17.3	0.18
	Positive	51	72.9	86	82.7	
$pAkt^{308}$	Negative	31	43.6	56	53.8	0.10
	Positive	40	56.4	48	46.2	

N, Number; *WT*, Wild-type

Discussion

In the existing literature an important number of papers focused on biochemical methods in order to evaluate the expression of p85 PI3K subunit in several tissues and to highlight its role in the regulation of fundamental cellular processes, such as the cell cycle and cell survival [20–23]. On the opposite, to our knowledge, the immunohistochemical expression of p85 protein has only been studied twice [24, 25]. In one of these studies the level of expression of three PI3K isoforms including p85 has been analyzed in a wide range of normal human tissues. All three isoforms were found to have differences in their spatial location and expression and were also specific for concrete tissues, a finding that might reflect the existence of tissue specific ligands related to the function of the above enzymes. In the aforementioned study, the immunohistochemical expression of p85 in breast tissue was found to be weakly positive only to areas featuring apocrine metaplasia, while normal breast lobules expressed no p85. It has been hypothesized that this might be the result of a very low level of expression of p85 under normal conditions or the predominance of several PI3K isoforms in breast tissue [25]. On the other hand, in breast carcinoma, an increased expression of p85 as compared to the adjacent normal mammary gland was observed using Western blotting with monoclonal antibodies [26].

Several questions have emerged. Is the increased expression of p85 in neoplastic tissue the result of its activation by

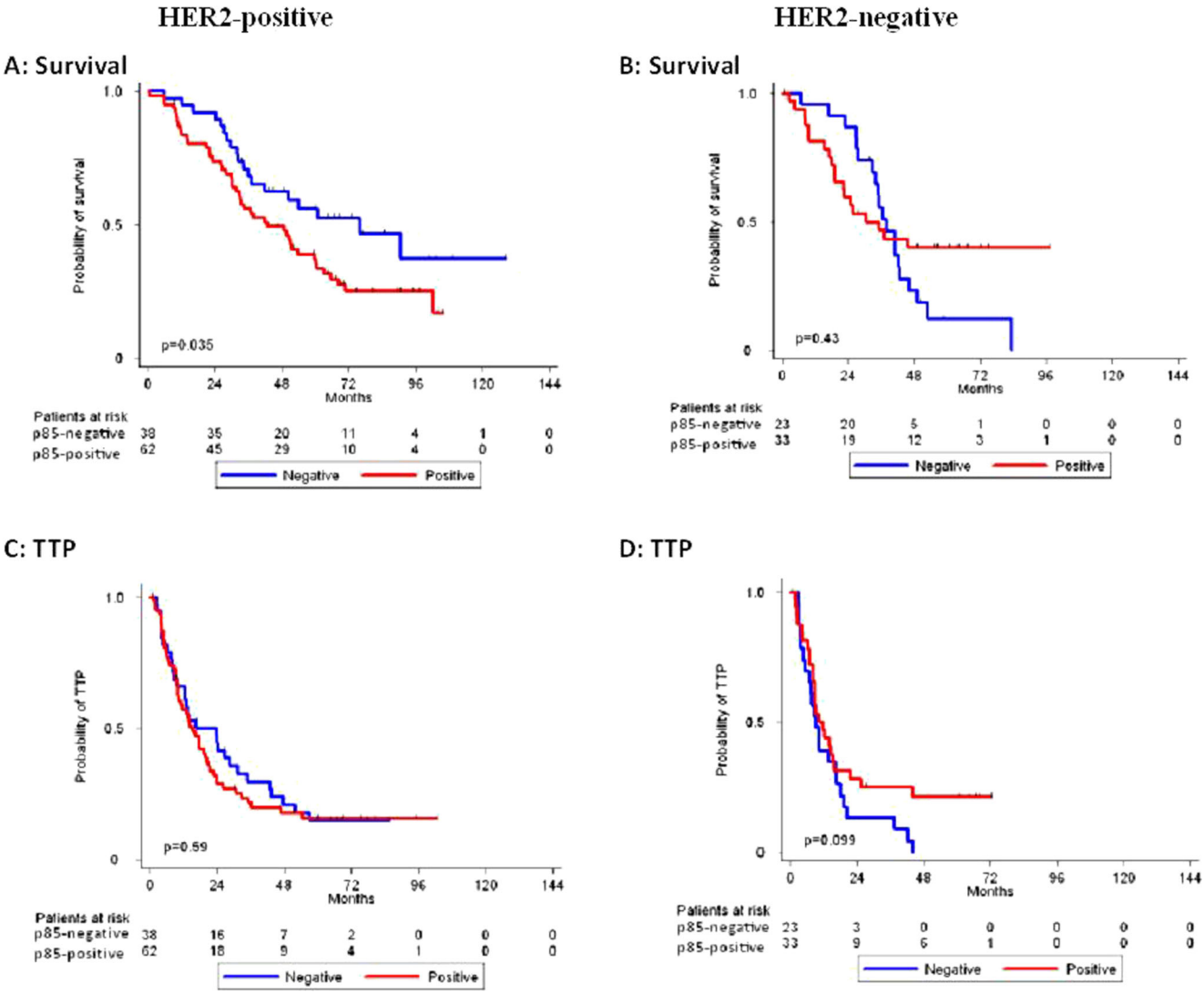

Fig. 2 Kaplan-Meier curves for survival (**a** and **b**) and time to progression (TTP) (**c** and d) according to p85 protein expression in HER2-positive (**a** and **c**) and HER2-negative (**b** and d) patients. Comparisons were made using the log-rank test

transmembrane tyrosine kinase activators? Could activated p85 be related to changes in the immunohistochemical expression of other components of the PTEN/PI3K/Akt pathway? Would all the above reflect differences in the biologic behavior of the tumors? The aforementioned questions led us to conduct the present study, in an effort to evaluate whether protein expression of p85 in a series of breast carcinomas could provide insight into molecular interactions of the PTEN/PI3K/Akt pathway and probably create perspectives for future targeted therapeutic interventions.

Our study has limitations, mostly related to the absence of similar immunohistochemical studies of p85 in breast cancer. Therefore the cut-off point that we used was rather arbitrary. Nevertheless, since as already stated, the p85 protein has not been found to be expressed by immunohistochemistry in normal breast tissue, we felt that it would be reasonable to consider any percentage or intensity of p85 observed in our tumors as positive expression.

In the present study no association was found between the up-regulation of p85, as shown by its immunohistochemical expression, and any of the other parameters under investigation, such as histological grade, hormonal status, components of the PTEN/PI3K/Akt pathway and HER2 status of the patients. The latter finding is consistent with the results of other investigators indicating that up-regulation of p85 is the result of activation not only of HER2 but also of several other transmembrane tyrosine kinase receptors, while other novel mechanisms might also contribute to the increase in the protein level of p85 [7, 27, 28].

The most important finding of our study was a statistically significant poor survival of HER2-positive patients featuring p85 protein expression, in spite of the tailored anti-HER2

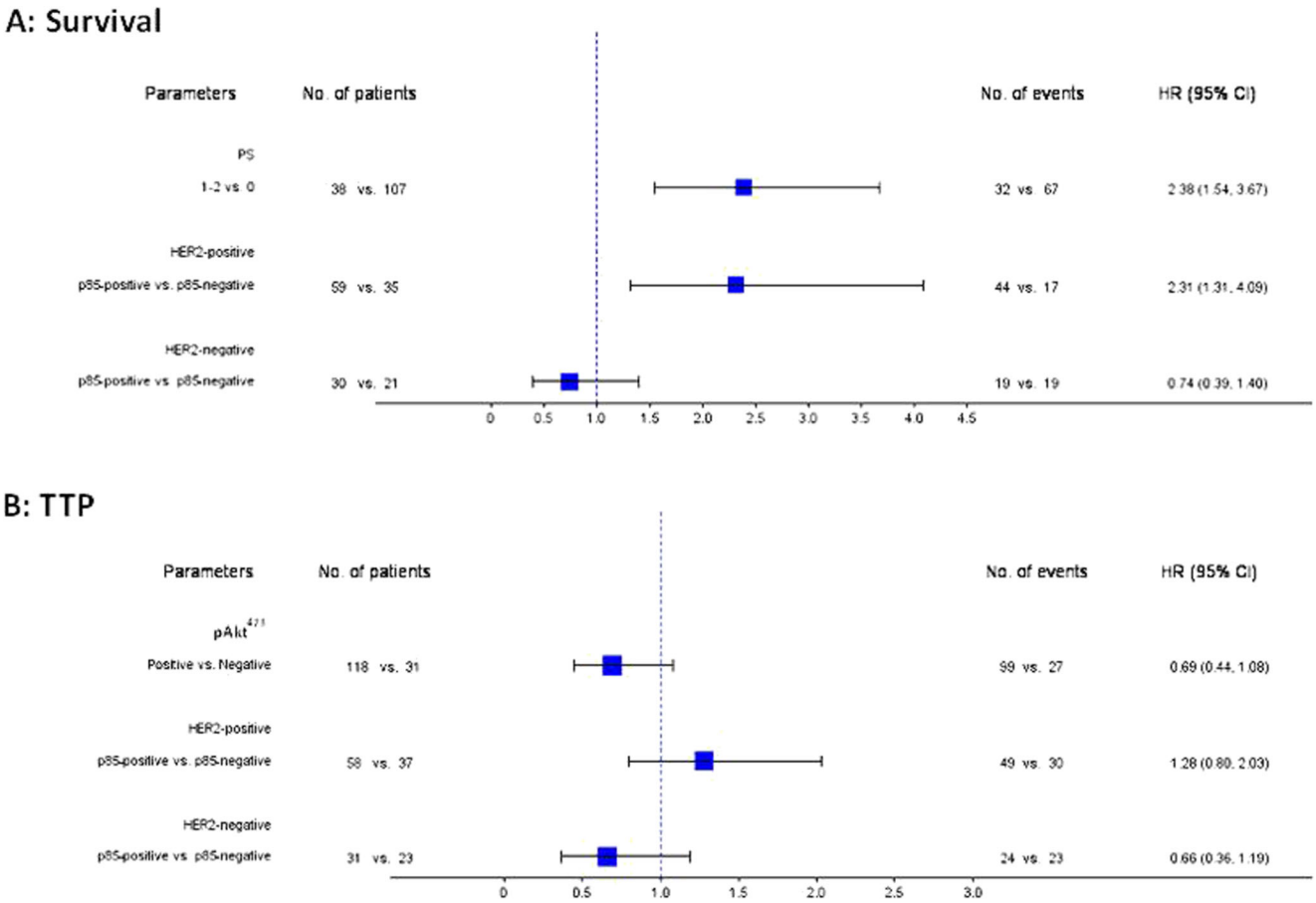

Fig. 3 Multivariate Cox regression analysis for survival (**a**) and time to progression (TTP) (**b**) presented by forest plots

treatment. Since all patients had been treated with trastuzumab it would be of great interest to investigate whether p85 up-regulation would interfere with mechanisms related to resistance to trastuzumab. Indeed one of the functions that are required for trastuzumab action is inhibition of post receptor signal transduction molecules [29]. One of these molecules is the p85 subunit of PI3K, which is constitutively associated with HER3. It has been demonstrated that in cells resistant to trastuzumab, activation of the PI3K/Akt pathway depends on the ErbB signaling pathway via tyrosine phosphorylation of HER3 [30]. As a consequence activation of p85 might result in trastuzumab resistance; it is uncertain however, whether at the tissue level, activation of p85 is expressed by a positive immunohistochemical reaction.

Analysis of the HER2-positive patients in an earlier report [11], revealed that a reduction in PTEN protein levels together with an increase in the incidence of *PIK3CA* mutations, irrespectively of the expression of p85, was associated with both decreased TTP and survival. In the same group of patients, despite activating alterations of components of the PI3K pathway, no over expression of $pAkt^{473}$ and $pAkt^{308}$ was observed. Indeed, increased PI3K but not Akt seems to be a widespread phenomenon in human breast cancer, indicating the different roles of the two components of one signaling pathway [26].

In HER2-negative patients the effect of treatment with trastuzumab was not influenced by increased p85 protein expression. It was of particular interest to examine this HER2-negative subgroup, since it is unlikely that such a population will ever be available in the future, as HER2 testing methods have significantly improved and cost regulation and established treatment guidelines would prevent the use of trastuzumab in HER2-negative patients. One could have postulated that in a setting of increased p85 protein expression, trastuzumab could have exerted a therapeutic effect in a "HER2-borderline" population, or even that trastuzumab could have an effect that would possibly be unrelated to HER2 and more dependent on downstream molecules. There was a recent review suggesting that, as HER2 positivity is more significant in stem cells, in the adjuvant setting, trastuzumab might be active in HER2 1+ or 2+ cases, as well [31]. Our study however, was not designed to support such hypotheses; it appears therefore, that at least in the metastatic setting, HER2-positive breast cancer patients treated with trastuzumab have a longer survival and TTP, however statistically not significant in this study, than similarly treated breast cancer patients with HER2-negative disease.

Our study is fraught with the disadvantages of all retrospective analyses, especially those that come from a non-trial population. Our results should thus be considered as hypothesis

generating, while results of prospective studies, such as the NEO-ALTO and ALTO studies, are eagerly awaited to clarify the field. Meanwhile the scientific and clinical communities have to establish optimal ways to evaluate p85 protein expression and standardize the methods and cut-offs.

In conclusion, the immunohistochemical expression of the p85 subunit of PI3K in advanced breast cancer was associated with poor survival in this trastuzumab-treated cohort of HER2-positive advanced breast cancer patients. The present finding highlights a possible role of p85 as a prognostic marker, which, if validated, might have important implications in the treatment of HER2-positive breast cancer patients.

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Competing Interests The authors declare that they have no competing interests. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Ethical Approval The translational research protocol has been approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Medicine (Protocol # 4283; January 14, 2008) under the general title "Investigation of major mechanisms of resistance to treatment with trastuzumab in patients with metastatic breast cancer". All patients included in the study after 2005 provided written informed consent for the provision of biological material for future research studies, before receiving any treatment. Waiver of consent was obtained from the Bioethics Committee for patients included in the study before 2005.

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