

ORIGINAL PAPER

PROGNOSTIC VALUE OF *PIK3CA* MUTATION STATUS, PTEN AND ANDROGEN RECEPTOR EXPRESSION FOR METASTASIS-FREE SURVIVAL IN HER2-POSITIVE BREAST CANCER PATIENTS TREATED WITH TRASTUZUMAB IN ADJUVANT SETTING

AGNIESZKA ADAMCZYK¹, JOANNA NIEMIEC¹, ANNA JANECKA¹, AGNIESZKA HARAZIN-LECHOWSKA², ALEKSANDRA AMBICKA², ALEKSANDRA GRELA-WOJEWODA³, MAŁGORZATA DOMAGAŁA-HADUCH³, IDA CEDRYCH³, KAJA MAJCHRZYK¹, ANNA KRUCZAK², JANUSZ RYS², JERZY JAKUBOWICZ⁴

¹Department of Applied Radiobiology, Maria Skłodowska-Curie Memorial Cancer Centre and Institute of Oncology, Krakow Branch, Krakow, Poland

²Department of Tumor Pathology, Maria Skłodowska-Curie Memorial Cancer Centre and Institute of Oncology, Krakow Branch, Krakow, Poland

³Department of Systemic and Generalized Malignancies, Maria Skłodowska-Curie Memorial Cancer Centre and Institute of Oncology, Krakow Branch, Krakow, Poland

⁴Department of Radiation Oncology, Maria Skłodowska-Curie Memorial Cancer Centre and Institute of Oncology, Krakow Branch, Krakow, Poland

Resistance to trastuzumab in patients with HER2-overexpressing breast cancer is associated with higher risk of progression or cancer death, and might be related to activation of PI3K/AKT/mTOR and Ras/Raf/MAPK signaling cascades and a decreased level of their inhibitor (PTEN).

HER2-overexpressing breast cancer patients (n = 75) treated with radical local therapy and trastuzumab in adjuvant setting were included into the study. Deoxyribonucleic acid isolated from paraffin sections was used to assess mutational status of the *PIK3CA* gene (p.H1047R and p.E545K mutations) by the quantitative polymerase chain reaction technique. Expression of selected proteins (ER, PgR, AR, Ki-67, EGFR) was assessed using immunohistochemistry.

In the studied group we found significantly higher Ki-67LI in EGFR-positive carcinomas (p = 0.048). Moreover, EGFR immunonegativity was observed more frequently in low-grade (G1/G2) carcinomas as well as in estrogen/progesterone and androgen receptor immunopositive tumors (p = 0.042, p = 0.016, p = 0.044, respectively). Favorable metastasis-free survival was observed in patients with pN0 and pN1 (vs. pN2+3) stage (p = 0.040) and with tumors characterized by low Ki-67LI (≤ 50% vs. > 50%) (p = 0.014). Patients with tumor androgen receptor immunonegativity (weak or lack of expression) or strong PTEN expression survived 3 years without metastases (p = 0.007).

The results of our study suggest that androgen receptor and PTEN status might be considered as indicators of trastuzumab sensitivity.

Key words: HER2-overexpressing breast cancer, PTEN, *PIK3CA* mutations.

Introduction

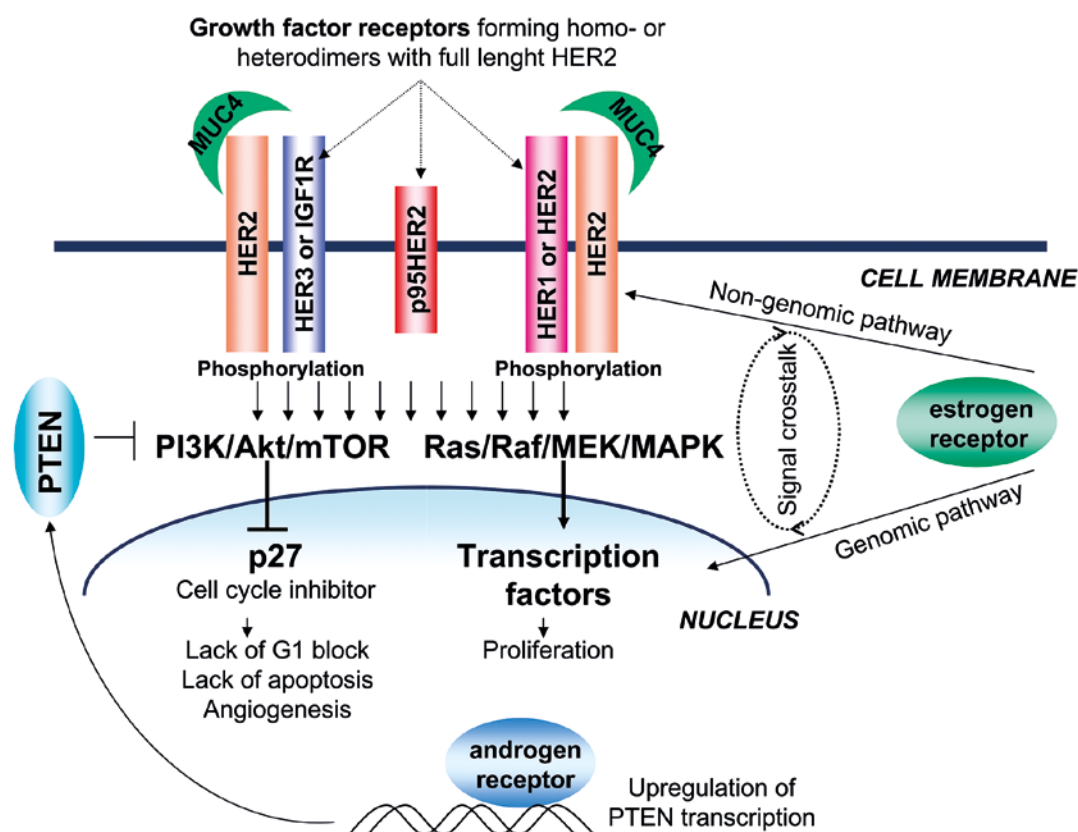
Overexpression of human epidermal growth factor receptor 2 (HER2) is noted in 20-25% of invasive breast cancer cases and is associated with poor prognosis [1]. Clinical trials published so far have shown that trastuzumab changes the natural history of the disease and improves disease-free and overall survival [2, 3].

Currently, trastuzumab in adjuvant setting for radically treated HER2-overexpressing breast cancer patients is a standard approach [4]. However, it has been shown that only 10-34% of patients with advanced breast cancer respond to trastuzumab in monotherapy [5]. This suggests a reduced sensitivity to this drug in some patients.

Resistance to trastuzumab in breast cancer patients with HER2 overexpression is associated with higher risk of progression or cancer death. Trastuzumab is a monoclonal antibody targeting the extracellular domain of HER2 protein and blocking signaling cascades triggered by HER2 (PI3K/AKT/mTOR and Ras/Raf/MAPK), this cascade, on the other hand, blocks p27-dependent cell cycle arrest and apoptosis. Additionally, the aforementioned cas-

cade is controlled by PTEN (phosphatase and tensin homolog deleted on chromosome ten) protein, which inhibits PI3K/Akt cascade [6-11]. The efficacy of trastuzumab may be limited by the following biological mechanisms:

- overexpression of MUC4 protein [6, 7, 9] (Fig. 1);
- activation of HER2 signaling cascades (PI3K/AKT/mTOR and/or Ras/Raf/MAPK) by alternative mechanisms: (a) interaction of receptor for insulin-like growth factor (IGF-1R) with HER2 [6-9, 12] (Fig. 1); (b) heterodimerization of HER2 with EGFR (epidermal growth factor receptor) family proteins (EGFR3, EGFR4, EGFR1) [6, 8, 9, 11-13] (Fig. 1); or (c) MET receptor [9]; (d) occurrence of truncated HER2 protein (p95HER2) constitutively activating signaling cascades [7-9, 13, 14]. The technique used for HER2 evaluation for diagnostic purposes detects the intracellular domain of HER2, but trastuzumab targets the extracellular domain. Therefore, the presence of truncated proteins could lead to resistance to trastuzumab even in the group with immunohistochemically detected HER2 overexpression;
- continuous stimulation of HER2-activated signaling cascades by HER2-independent mechanisms:



HER2 – epidermal growth factor receptor 2; p95HER2 – truncated HER2; IGF1R – insulin-like growth factor 1 receptor; PTEN – phosphatase and tensin homolog

Fig. 1. Signaling cascades activated by HER2 and mechanisms involved in their control as potential mechanisms of trastuzumab resistance [1-17, 34]

(a) inactivating mutation or loss of PTEN protein which makes HER2/HER3/Akt cascade continuously active [6-13, 15] (Fig. 1); (b) *PIK3CA* (phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit) mutations (in exon 9 and 20) resulting in PI3K (phosphatidylinositol-3-kinase) protein activation [6-12, 15-17] (Fig. 1) or (c) reduced expression of p27 protein (cell cycle inhibitor), which abolishes the trastuzumab-induced block in the G1 phase [6, 9-11] (Fig. 1);

- the interaction of signaling cascades triggered by HER2 with estrogen, progesterone or androgen receptor (Fig. 1).

Therefore, in our preliminary study, we analyzed the prognostic value of *PIK3CA* mutation status, PTEN and androgen receptor expression for metastasis-free survival in HER2-positive breast cancer patients treated with trastuzumab in adjuvant setting.

Material and methods

Patients

The studied group consisted of 75 patients with invasive ductal breast cancer (T \geq 1, N \geq 0, M0) with overexpression of HER2, who underwent radical surgery between 2007 and 2011 at the Department of Surgical Oncology, Centre of Oncology, Cracow Branch. None of the patients received neoadjuvant chemotherapy or radiotherapy. The mean age of patients was 53.1 ± 1.0 (mean \pm SE) (range 31–69) years. All patients received adjuvant chemotherapy based on anthracyclines (doxorubicin, 54 patients) or anthracyclines and taxanes (doxorubicin, docetaxel, 20 patients) and trastuzumab in adjuvant setting. Some patients received radiotherapy, as individually indicated. Hormonal therapy was applied in 38 patients with tumors presenting estrogen/progesterone receptor (ER/PR) expression. The Ethical Committee at the Regional Medical Chamber in Krakow approved the study (decision dated 4 December 2013).

No specific consent was needed for this study as it was a retrospective study performed on archived tissues with no direct patient contact, no modification of diagnostic or treatment procedures and no personal patients' data revealed.

Material

Archival specimens from primary tumor and synchronous lymph node metastasis (if present) were reexamined independently by two pathologists (A.H-L., A.A.) to confirm the histological diagnosis and tumor grade.

Immunohistochemical staining

Status of ER, PR, HER2 expression was evaluated during diagnostic procedures and data were retrieved from patients' files. Expression of the estrogen (ER) or progesterone (PR) receptor in $> 1\%$ of tumor cells was considered as immunopositivity. Overexpression of HER2 was tested using immunohistochemistry (IHC) (HercepTest, Dako Denmark A/S, Glostrup Denmark) and, in case of an unclear result (expression assessed as 2+), amplification of the *HER2* gene was verified using fluorescence *in situ* hybridization (FISH) – PathVysion HER2 DNA Probe (Abbot Molecular). Finally, overexpression of HER2 protein/amplification of *HER2* gene was established according to recommended standards [18].

For estimating PTEN, androgen receptor (AR), EGFR and Ki67 expression, sections from formalin-fixed paraffin embedded tissues were cut at $4 \mu\text{m}$, mounted on SuperFrost Plus (Menzel-Gläser, Germany) slides, and then deparaffinized and hydrated through a series of xylenes and alcohols.

After antigen unmasking procedures (Table I), slides were incubated for 30 min. in $0.3\% \text{H}_2\text{O}_2$ diluted in methanol. Non-specific binding of antibodies was blocked for 5 min. Incubation was performed with UltraVision Protein Block (Thermo Scientific, Fremont, USA). After incubation with primary anti-

Table I. Detailed information on immunohistochemical procedures.

ANTIGEN	CLONE	MANUFACTURER	ANTIGEN RETRIEVAL	DILUTION	NUMBER OF STAINED CASES
PTEN	138G6	Cell Signaling ¹	TRS, pH = 6.1 DAKO ² , 50 min, 96°C	1 : 75 ^a	67
Ki-67	MIB-1	DAKO ²		1 : 100 ^b	68
AR	SP107	Cell Marque ³		1 : 100 ^b	70
EGFR	H11	DAKO ²	Proteinase K ² , 10 min, RT	1 : 200 ^b	70

¹Cell Signaling, Danvers, USA

²DakoCytomation Denmark A/S, Glostrup, Denmark

³Cell Marque, Rocklin, USA

Incubation with primary antibody: ^a1 h at 37°C; ^bovernight, 4°C;

body (for details see Table I), for protein visualization, sections were treated with the BrightVision detection system (Immunologic, Duiven, The Netherlands) and DAB (Vector Laboratories, Inc., Burlingame, USA). Hematoxylin was used for nuclear counterstaining. Each step of the staining procedure was followed by washing in tris-buffered saline and Tween 20 (TBST).

We did not obtain IHC results for all proteins in all cases (the number of cases is shown in Table I) because of an insufficient amount of tissue in paraffin blocks or small fragments of tumor tissue that hindered obtaining reliable results.

IHC Evaluation

IHC stainings were evaluated exclusively in the invasive component of the tumors. We applied the following criteria for protein immunopositivity: (1) EGFR – expression in more than 1% of tumor cells, (2) AR immunopositivity (Fig. 2A) – more than 50% of cells with weak staining or any percentage with moderate/strong staining (3) PTEN – any percentage of cells with strong PTEN expression (clearly distinguished from stroma) (Fig. 2B). Ki-67 labeling index (Ki-67LI) was assessed in about 5 high power fields in > 1000 tumor cells and was calculated as the percentage of cells with nuclear Ki-67 immunopositivity. Membranous Ki-67 expression (detected with MIB-1 monoclonal antibody) was not included in Ki-67LI assessment.

In case of all proteins, with the exception of Ki-67, a binary scale was used to evaluate staining: 0 – negative, 1 – positive.

Quantitative polymerase chain reaction analysis of PIK3CA mutation status

DNA extraction and spectrophotometric measurements

For each patient, DNA isolation was performed using two $5 \mu\text{m}$ thick sections from formalin-fixed, par-

affin-embedded tumor tissue blocks. For each sample a fresh microtome blade was used and both the microtome and the work area were cleaned. DNA was extracted using the ReliaPrep FFPE gDNA Miniprep System from Promega Corp. (Madison, WI 53711 USA). All extractions were performed manually according to the manufacturer's protocol.

The purity and concentration of isolated DNA were assessed using BioPhotometer plus (Eppendorf, Hamburg, Germany) with TrayCell (Hellma, Müllheim, Germany) according to the manufacturer's instructions. Measurements were performed in duplicate (to eliminate false results caused by some debris or bubbles) and the mean value of total DNA yield was calculated. The A260/A280 (indicating protein and RNA contamination) ratio was established. An A260/A280 ratio in the range of 1.8–2.0 is considered as an indicator of pure DNA, but for real-time PCR, according to the brochure of TaqMan Mutation Detection Assay, DNA specimens with A260/A280 ratio > 1.7 are considered satisfactory. In our series all specimens had sufficient DNA amount and purity.

Real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was performed using the ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). For each patient, on the same plate, three different assays were performed: (i) PIK3CA 775 TaqMan[®] Mutation Detection Assay (Assay ID Hs00000831_mu) to assess the H1047R PIK3CA mutation status, (ii) PIK3CA 763 TaqMan[®] Mutation Detection Assay (Assay ID Hs00000824_mu) to assess the E545K PIK3CA mutation status, (iii) PIK3CA TaqMan Mutation Detection Reference Assay (Assay ID Hs00001025_rf) to detect the conservative fragment of the PIK3CA gene. All assays were manufactured by Applied Biosystems (Foster City, CA, USA). Each well contained the following reagents: $2 \mu\text{l}$ of one of the above-mentioned

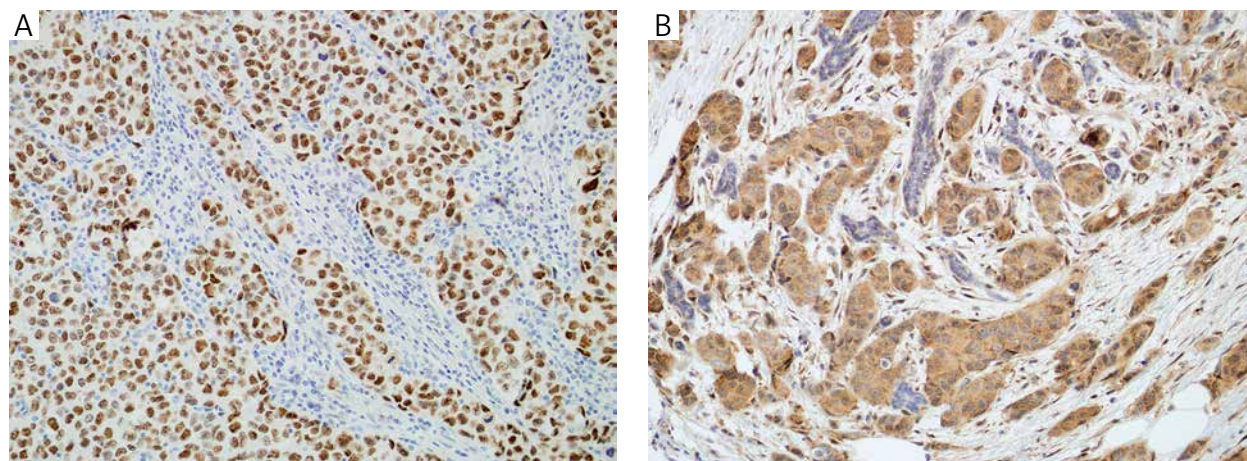


Fig. 2. Expression of: A) androgen receptor (AR) and B) PTEN in breast cancer cells

Table II. *PIK3CA* mutation status and protein expression observed in investigated continuous (Mann-Whitney test) or categorical variables (Pearson χ^2 test)

PARAMETER	CATEGORY	ALL	KI-67LI	PIK3CA MUTATIONS***				EGFR				PTEN				AR			
				N (%)	(N)	MEAN \pm SD	- N (%)***	+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	- N (%)	+ N (%)	
T	1	28	(26)	30.0 \pm 14.0	24 (85.7)	4 (14.3)	20 (76.9)	6 (23.1)	14 (60.9)	9 (39.1)	14 (60.9)	9 (39.1)	8 (29.6)	19 (70.4)					
	2	45	(44)	33.5 \pm 12.5	38 (84.4)	7 (15.6)	41 (89.1)	5 (10.9)	37 (82.2)	8 (17.8)	37 (82.2)	8 (17.8)	6 (13.6)	38 (86.4)					
pN	0 + 1	60	(57)	32.2 \pm 12.3	50 (83.3)	10 (16.7)	49 (83.1)	10 (16.9)	41 (75.9)	13 (24.1)	41 (75.9)	13 (24.1)	12 (20.7)	46 (79.3)					
	2 + 3	14	(14)	31.3 \pm 16.0	13 (92.9)	1 (7.1)	13 (92.9)	1 (7.1)	10 (71.4)	4 (28.6)	10 (71.4)	4 (28.6)	2 (14.3)	12 (85.7)					
G	1 + 2	1 + 23	(24)	28.4 \pm 12.1	21 (84.0)	4 (16.0)	25 (96.1)	1 (3.9)	15 (71.4)	6 (28.6)	15 (71.4)	6 (28.6)	2 (7.7)	24 (92.3)					
	3	49	(47)	33.9 \pm 13.2	42 (85.7)	7 (14.3)	37 (78.7)	10 (21.3) ^b	36 (76.6)	11 (23.4)	36 (76.6)	11 (23.4)	12 (26.1)	34 (73.9)					
ER/PR	-	36	(35)	31.7 \pm 13.3	31 (86.1)	5 (13.9)	26 (74.3)	9 (25.7)	25 (75.8)	8 (24.2)	25 (75.8)	8 (24.2)	8 (22.9)	27 (77.1)					
	+*	38	(36)	32.4 \pm 12.9	32 (84.2)	6 (15.8)	36 (94.7)	2 (5.3) ^c	26 (74.3)	9 (25.7)	26 (74.3)	9 (25.7)	6 (16.2)	31 (83.8)					
AR	-	15	(15)	28.9 \pm 12.8	13 (86.7)	2 (13.3)	10 (66.7)	5 (33.3)	13 (92.9)	1 (7.1)	13 (92.9)	1 (7.1)							
	+	55	(55)	32.4 \pm 13.0	46 (83.6)	9 (16.4)	51 (89.5)	6 (10.5) ^d	38 (70.4)	16 (29.6)	38 (70.4)	16 (29.6)							
PTEN	-	50	(50)	34.0 \pm 13.2	41 (82.0)	9 (18.0)	43 (82.7)	9 (17.3)											
	+	17	(17)	27.7 \pm 12.2	15 (88.2)	2 (11.8)	15 (93.7)	1 (6.3)											
EGFR	-	59	(59)	30.7 \pm 11.5	50 (84.7)	9 (15.3)													
	+	11	(11)	38.8 \pm 19.3 ^a	9 (81.8)	2 (18.2)													
PIK3CA mutations **	-	64	(58)	31.9 \pm 13.3															
	+	11	(10)	31.5 \pm 14.4															

PIK3CA: Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit; ER: estrogen receptor; PR: progesterone receptor; AR: androgen receptor; PTEN: phosphatase and tensin homolog deleted on chromosome ten; EGFR: epidermal growth factor receptor.

*: "+" identifies ER or PR immunopositive cases, "-" - ER and PR immunonegative ones; ** "-" - tumours without H1047R or E545K PIK3CA mutations "+" tumours with H1047R or E545K PIK3CA mutation; ***, percentage from the row

^ap = 0.048; ^bp = 0.042; ^cp = 0.016; ^dp = 0.044

TaqMan® Mutation Detection Assays, 4 µl (exactly 20 ng) of genomic DNA isolated from FFPE tissue, 10 µl of TaqMan Genotyping Master Mix (Applied Biosystems, Foster City, CA, USA), 0.4 µl of Exogenous IPC Template DNA, 2 µl of Exogenous IPC Mix (Applied Biosystems, Foster City, CA, USA) and 1.6 µl of nuclease-free water suitable for PCR (Ambion, Austin, TX, USA). The final reaction volume was 20 µl per well. Thermocycling conditions were set according to TaqMan Mutation Detection Assays recommendations: initial denaturation – 95°C, 10 minutes; 5 cycles: 92°C, 15 seconds and 58°C for 1 minute; 40 cycles: 92°C for 15 seconds and 60°C for 1 minute. Each processing plate contained 2-3 wells in which nuclease-free water was added instead of DNA – no template controls (NTCs).

According to the manufacturer, the Ct value determined for a gene reference assay should be in the 18-28 range for 20 µl of reaction volume.

Data obtained from qPCR reaction were analyzed using Mutation Detector Software to determine the presence or absence of *PIK3CA* mutations.

Statistical analysis

Descriptive statistics were used to determine mean values and standard errors of means (SE). Relationships between categorical variables were analyzed using Fisher's test for 2 × 2 tables. Differences between groups were assessed using the Mann-Whitney test. Metastasis-free survival (MFS) was defined as the time from surgery to the clinically or radiographically confirmed presence of metastases. The probability of survival was calculated using the Kaplan-Meier method. The log-rank test was used to investigate the statistical significance of the differences observed between groups and to establish a cut-off point for continuous variables. In all statistical procedures, $p < 0.05$ was considered significant. STATISTICA v.10 software (StatSoft, Inc., Tulsa, OK, USA) was used for calculations.

Results

Relations between *PIK3CA* mutations, PTEN, Ki-67, AR, EGFR, ER/PgR expression and clinical parameters

In the investigated group, in 36 (48.6%) tumors expression of both estrogen and progesterone receptors was not detected. Fifty-five tumors were classified as AR positive (78.6%). Strong PTEN expression was detected in 17 (25.4%), while EGFR immunopositivity – in 11 (15.7%) cases. More detailed data are presented in Table II.

In the studied group we identified 9/75 (12%) tumors with the H1047R mutation and 2/75 (2.7%) with the E545K mutation in the *PIK3CA* gene. For

further analysis we separated the group of patients with at least one *PIK3CA* mutation.

EGFR immunonegativity was significantly related to lower Ki-67LI, lower tumor grade (G1 + G2) as well as to AR and ER/PR immunopositivity (Table II). Higher Ki-67LI was observed in G3 than in G1+G2 carcinomas (Table II). The aforementioned relationship did not reach statistical significance ($p = 0.064$), but it is worth mentioning that there was only one G1 tumor.

In our series the mean and median value for Ki-67LI was 31.9 ± 1.5 (mean \pm SE).

Survival analysis

Survival analysis was conducted in a group of 74 patients. Mean time of follow-up was 45.7 months (range 11.4-85.3). For Ki-67LI, the cut-off point was established using the minimal p-value method from the log-rank test, because the median/mean value was not significant. A significant difference in patients' survival was found for Ki-67LI cut-off values ranging from 47% to 51%, however, for further analysis we selected 50% as the cut-off point. The above-mentioned cut-off point is high, but it should be mentioned that we analyzed a group of HER2-overexpressing carcinomas which generally are characterised by higher Ki-67LI. When a group encompassing all subtypes was analyzed, a lower cut-off point was established [19].

Favorable MFS was observed in patients with pN0 and pN1 (vs. pN2+3) stage ($p = 0.040$, Fig. 3A) and tumors characterized by low Ki-67LI ($\leq 50\%$ vs. $> 50\%$) ($p = 0.014$, Fig. 3B). In groups of AR immunonegative patients and PTEN immunopositive patients 100% MFS was observed, although it was on the border of statistical significance ($p = 0.135$; $p = 0.065$, respectively, Fig. 3C). However, patients with tumor androgen receptor immunonegativity (lack of or weak expression) or PTEN strong expression survived 3 years without metastases ($p = 0.007$, Fig. 3D). We were not able to assess the prognostic significance of AR/PTEN coexpression with Cox analysis, as there was no complete observation (metastases) in the low-risk group. In the above-described situation the Cox model does not work.

None of the other studied parameters such as grade, T, ER/PR, EGFR and *PIK3CA* mutation status statistically significantly influenced survival of breast cancer patients.

Discussion

Phosphatidylinositol 3 kinases (PI3Ks) encompass 3 subfamilies, although the most closely investigated is class I PI3K (mainly IA PI3K), which is involved in cell growth, proliferation and survival [20]. Class IA

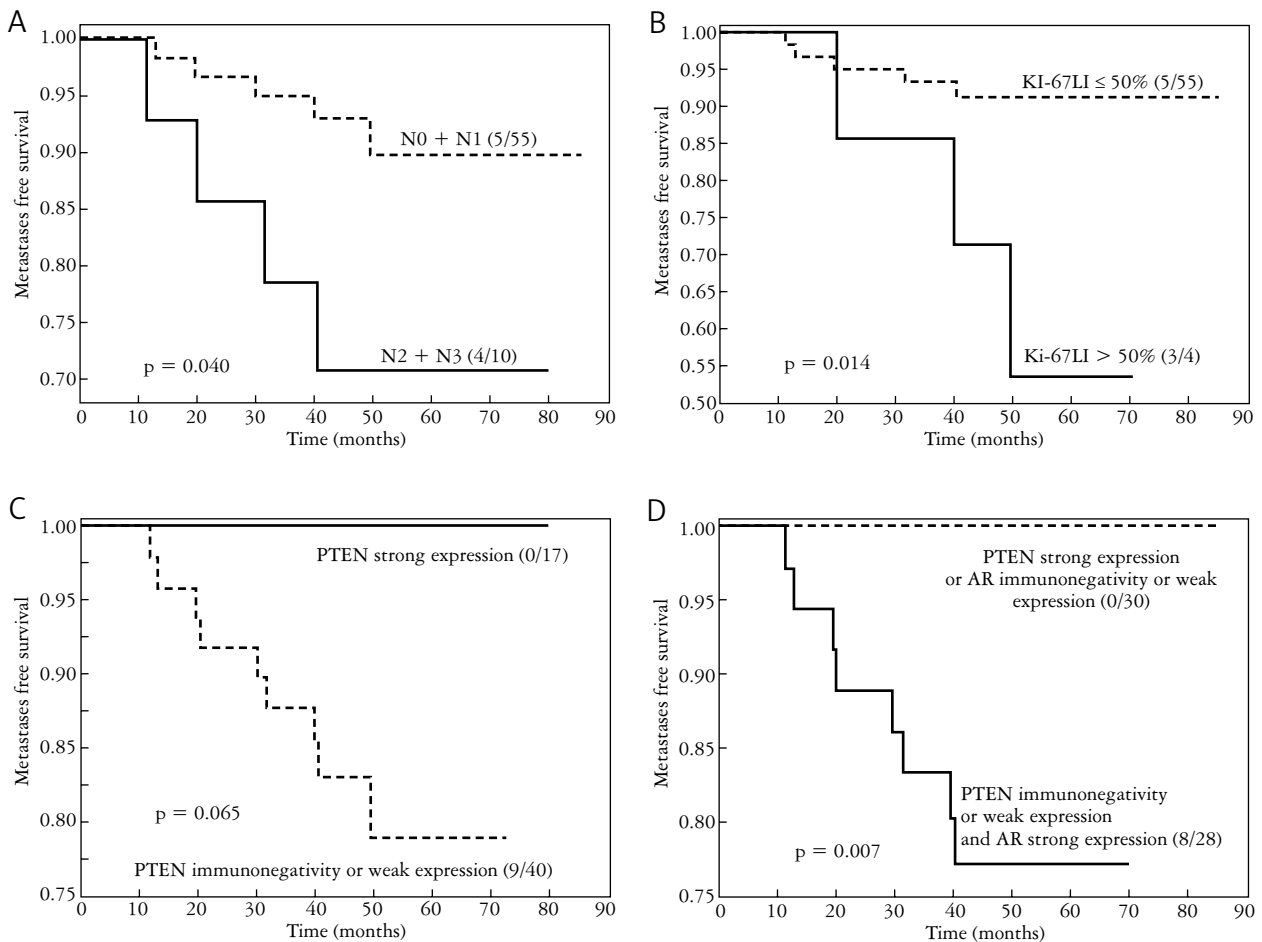


Fig. 3. Disease-free survival of patients with invasive ductal breast cancer stratified by: A) nodal status, B) Ki67 labelling index, C) PTEN expression, D) combined PTEN and androgen receptor (AR) expression

PI3K is a heterodimer of the p85 regulatory subunit and the p110 catalytic subunit. There are three forms of the p110 catalytic subunit coded by *PIK3CA*, *PIK3CB* and *PIK3CD*. Mutations and an increased copy number of the *PIK3CA* gene have been found in various human malignancies [20]. About 80% of *PIK3CA* mutations take place within helical (E542K, E545K) and kinase (H1047R) domains, which leads to increased catalytic activity – production of phosphatidylinositol (3,4,5) triphosphate (PIP3) and finally to enhanced cell proliferation and survival [21]. It is estimated that about 40% of breast cancer with positive ER receptors also carry activating *PIK3CA* mutations [21]. In our study we were able to detect 9/75 (12%) tumors with the H1047R mutation and 2/75 (2.7%) with the E545K mutation in the *PIK3CA* gene. Our results are comparable to other studies, in which the mutated *PIK3CA* gene was found in 16%–24% of cases [22–28]. Comparing data with results obtained by other authors sometimes could be problematic because different mutations are investigated in groups of cancer patients with different clinical characteristics. Moreover, in different studies, the

mutational status of the *PIK3CA* gene was studied using different material such as serum [29, 30], paraffin embedded formalin fixed tissues or frozen tissues [17, 22–24, 31, 32].

We, similarly to other studies [24, 26, 28], did not find an association between *PIK3CA* mutation status and other clinical and biological parameters. However, there are reports in which the correlation between *PIK3CA* mutations and grade [26] or nodal status (in the ER-positive group) [27] was reported.

It is expected that *PIK3CA* mutations might influence effects of trastuzumab treatment – firstly because activating mutations alter cell proliferation and survival, and secondly because PI3K is essential in signaling cascades triggered by HER2. However, the relation between mutation status of *PIK3CA* and breast patients' survival is controversial. It may be due to different groups investigated, or various treatment regimens implemented. Some authors found that patients with tumors bearing an activating *PIK3CA* mutation are characterized by shorter time to recurrence or progression [29, 32], while others reported that patients with *PIK3CA* mutations were

less likely to have a pathologic complete response [23, 25, 31]. However, other studies did not confirm the relationship between *PIK3CA* mutations and patient survival (disease/progression/recurrence-free [12, 17, 24], or overall [22]) or reaction to trastuzumab treatment [22, 30]. But, as mentioned before, the above-discussed studies included groups with different treatment schedules (neoadjuvant [23, 25, 31] or adjuvant [29, 24, 26] setting) or different clinical characteristic (metastatic breast cancer [30, 32] or patients without distant metastases [25, 29]). It seems that *PIK3CA* mutation status is more valuable as a predictive factor for metastatic patients and neoadjuvant trastuzumab treatment.

Very often alongside *PIK3CA* mutation status, PTEN expression is investigated. This is not surprising, as PTEN is a phosphatase and an antagonist of PI3K. PTEN detaches 3rd phosphate from PIP3 and disturbs downstream signaling of activated PI3K. In our study, 75% of tumors were classified as low PTEN expressing. Other authors achieved results in a wide range from 18 to 52% [12, 17, 22, 32]. We discovered that patients with HER2-positive tumors with strong PTEN expression had a 100% survival rate. Some authors reported no relationship between PTEN loss in HER2-positive tumors and response to trastuzumab treatment [22, 33], overall [33] as well as relapse/metastasis-free survival [32, 33]. In other studies shorter survival for individuals with HER2 overexpressing tumors with PTEN loss [22, 32] or shorter time to progression [12] was noted. Moreover, patients with reduced PTEN expression were less likely to achieve a pathological complete response [23]. All the above-presented discrepancies could result from differences in methodological aspects or different clinical characteristic or treatment schedules of analyzed groups.

Data concerning the influence of PTEN and *PIK3CA* are not unequivocal. However, PTEN and *PIK3CA* are only two out of many other points, in signaling cascades activated by HER2 (PI3K/AKT/mTOR and/or Ras/Raf/MAPK), which can be responsible for resistance to trastuzumab.

As AR has been suggested to upregulate PTEN transcription in breast cancer cells [34], we also assessed AR status. In our study, strong AR positivity was found in 78.6% of cases. In a meta-analysis comprising 7693 patients, in ER-positive tumors, 74.8% showed expression of AR, while among ER-negative cancers, AR expression was seen in 31.8% of cases. Among PR-positive tumors, 77.0% showed expression of AR, but for PR-negative cancers it was only 51.4% [35]. The discrepancy might be the effect of different clinical characteristics of the populations included in our study and studies eligible for meta-analysis. In our study, AR expression was more frequent among EGFR immunonegative cases. To the best of

our knowledge the aforementioned correlation was not studied elsewhere.

In our study, AR immunopositivity was an indicator of poor MFS in early breast cancer patients treated with trastuzumab in adjuvant setting. To the best of our knowledge, survival according to AR expression was not analyzed in the above-mentioned group of patients. Our results are consistent with results reported for male breast cancer, where AR positivity was related to shorter overall and disease-free survival [36]. However, contrary results are reported in the meta-analysis, which revealed that AR expression is related to favorable overall and disease-free survival both in ER-positive patients and in negative ones [35].

Conclusions

The results of our study suggest that AR and PTEN status might be considered as indicators of trastuzumab sensitivity in the group of HER2-positive breast cancer patients treated with trastuzumab in adjuvant setting.

The authors declare no conflict of interest.

The study was financed by the National Science Centre, based on decision DEC-2013/09/B/NZ5/00764. We would like to thank Anna Cichocka, MSc for her help during immunohistochemical procedures.

References

- Slamon DJ, Clark GM, Wong GS, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987; 235: 177-182.
- Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against her2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2001; 344: 783-792.
- Slamon D, Eiermann W, Robert N, et al. Breast Cancer International Research Group. Adjuvant trastuzumab in HER-2 positive breast cancer. *N Engl J Med* 2011; 365: 1273-1283.
- Romond EH, Perez EA, Bryant J, et al. "Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer". *N Engl J Med* 2005; 353: 1673-1684.
- Vogel CL, Cobleigh MA, Tripathy D, et al. Efficiency and safety of trastuzumab as a single agent in first line treatment of HER 2 overexpressing metastatic breast cancer. *J Clin Oncol* 2002; 20: 719-726.
- Nahta R, Yu D, Hung MC, et al. Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. *Nat Clin Pract Oncol* 2006; 3: 269-280.
- Nahta R, Esteva FJ. HER2 therapy: molecular mechanisms of trastuzumab resistance. *Breast Cancer Res* 2006; 8: 215.
- Wong AL, Lee SC. Mechanisms of Resistance to Trastuzumab and Novel Therapeutic Strategies in HER2-Positive Breast Cancer. *Int J Breast Cancer* 2012; 2012: 415170.
- Mukohara T. Mechanisms of resistance to anti-human epidermal growth factor receptor 2 agents in breast cancer. *Cancer Sci* 2011; 102: 1-8.

10. Rosen LS, Ashurst HL, Chap L. Targeting signal transduction pathways in metastatic breast cancer: a comprehensive review. *Oncologist* 2010; 15: 216-235.
11. Fedele P, Calvani N, Marino A, et al. Targeted agents to reverse resistance to endocrine therapy in metastatic breast cancer: where are we now and where are we going? *Crit Rev Oncol Hematol* 2012; 84: 243-251.
12. Gallardo A, Lerma E, Escuin D, et al. Increased signalling of EGFR and IGF1R, and deregulation of PTEN/PI3K/Akt pathway are related with trastuzumab resistance in HER2 breast carcinomas. *Br J Cancer* 2012; 106: 1367-73.
13. Han SW, Cha Y, Paquet A, et al. Correlation of HER2, p95HER2 and HER3 expression and treatment outcome of lapatinib plus capecitabine in her2-positive metastatic breast cancer. *PLoS One* 2012; 7: e39943.
14. Arribas J, Baselga J, Pedersen K, et al. p95HER2 and breast cancer. *Cancer Res* 2011; 71: 1515-1519.
15. Wang Y, Liu Y, Du Y, et al. The predictive role of phosphatase and tensin homolog (PTEN) loss, phosphoinositol-3 (PI3) kinase (PIK3CA) mutation, and PI3K pathway activation in sensitivity to trastuzumab in HER2-positive breast cancer: a meta-analysis. *Curr Med Res Opin* 2013; 29: 633-642.
16. Kataoka Y, Mukohara T, Shimada H, et al. Association between gain-of-function mutations in PIK3CA and resistance to HER2-targeted agents in HER2-amplified breast cancer cell lines. *Ann Oncol* 2010; 21: 255-262.
17. Wang L, Zhang Q, Zhang J, et al. PI3K pathway activation results in low efficacy of both trastuzumab and lapatinib. *BMC Cancer* 2011; 11: 248.
18. Wolff AC, Hammond ME, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *Arch Pathol Lab Med* 2007; 131: 18-43.
19. Niemiec J, Adamczyk A, Ambicka A, et al. BGX-Ki-67 Index as a Supplementary Marker to MIB-1 Index, Enabling More Precise Distinction Between Luminal A and B Subtypes of Breast Carcinoma and Eliminating the Problem of Membranous/Cytoplasmic MIB-1 Staining. *Am J Clin Pathol* 201; 143: 419-429.
20. Jiang BH, Liu LZ. PI3K/PTEN signaling in tumorigenesis and angiogenesis. *Biochim Biophys Acta* 2008; 1784: 150-158.
21. Mayer IA, Arteaga CL. PIK3CA activating mutations: a discordant role in early versus advanced hormone-dependent estrogen receptor-positive breast cancer? *J Clin Oncol* 2014; 32: 2932-1934.
22. Esteva FJ, Guo H, Zhang S, et al. PTEN, PIK3CA, p-AKT, and p-p70S6K status: association with trastuzumab response and survival in patients with HER2-positive metastatic breast cancer. *Am J Pathol* 2010; 177: 1647-1656.
23. Sueta A, Yamamoto Y, Yamamoto-Ibusuki M, et al. An integrative analysis of PIK3CA mutation, PTEN, and INPP4B expression in terms of trastuzumab efficacy in HER2-positive breast cancer. *PLoS One* 2014; 9: e116054.
24. Pogue-Geile KL, Song N, Jeong JH, et al. Intrinsic Subtypes, PIK3CA Mutation, and the Degree of Benefit From Adjuvant Trastuzumab in the NSABP B-31 Trial. *J Clin Oncol* 2015; 33: 1340-1347.
25. Loibl S, von Minckwitz G, Schneeweiss A, et al. PIK3CA mutations are associated with lower rates of pathologic complete response to anti-human epidermal growth factor receptor 2 (her2) therapy in primary HER2-overexpressing breast cancer. *J Clin Oncol* 2014; 32: 3212-3220.
26. Beelen K, Opdam M, Severson TM, et al. PIK3CA mutations, phosphatase and tensin homolog, human epidermal growth factor receptor 2, and insulin-like growth factor 1 receptor and adjuvant tamoxifen resistance in postmenopausal breast cancer patients. *Breast Cancer Res* 2014; 16: R13.
27. Liedtke C, Cardone L, Tordai A, et al. PIK3CA-activating mutations and chemotherapy sensitivity in stage II-III breast cancer. *Breast Cancer Res* 2008; 10: R27.
28. Arsenic R, Lehmann A, Budczies J, et al. Analysis of PIK3CA mutations in breast cancer subtypes. *Appl Immunohistochem Mol Morphol* 2014; 22: 50-56.
29. Oshiro C, Kagara N, Naoi Y, et al. PIK3CA mutations in serum DNA are predictive of recurrence in primary breast cancer patients. *Breast Cancer Res Treat* 2015; 150: 299-307.
30. Tolaney S, Burris H, Gartner E, et al. Phase I/II study of pилaralisib (SAR245408) in combination with trastuzumab or trastuzumab plus paclitaxel in trastuzumab-refractory HER2-positive metastatic breast cancer. *Breast Cancer Res Treat* 2015; 149: 151-161.
31. Majewski JJ, Nuciforo P, Mittempergher L, et al. PIK3CA mutations are associated with decreased benefit to neoadjuvant human epidermal growth factor receptor 2-targeted therapies in breast cancer. *J Clin Oncol* 2015; 33: 1334-1339.
32. Razis E, Bobos M, Kotoula V, et al. Evaluation of the association of PIK3CA mutations and PTEN loss with efficacy of trastuzumab therapy in metastatic breast cancer. *Breast Cancer Res Treat* 2011; 128: 447-456.
33. Gori S1, Sidoni A, Colozza M, et al. EGFR, pMAPK, pAkt and PTEN status by immunohistochemistry: correlation with clinical outcome in HER2-positive metastatic breast cancer patients treated with trastuzumab. *Ann Oncol* 2009; 20: 648-654.
34. Wang Y, Romigh T, He X, et al. Differential regulation of PTEN expression by androgen receptor in prostate and breast cancers. *Oncogene* 2011; 30: 4327-4338.
35. Vera-Badillo FE, Templeton AJ, de Gouveia P, et al. Androgen receptor expression and outcomes in early breast cancer: a systematic review and meta-analysis. *J Natl Cancer Inst* 2014; 106: djt319.
36. Wenhui Z, Shuo L, Dabei T, et al. Androgen receptor expression in male breast cancer predicts inferior outcome and poor response to tamoxifen treatment. *Eur J Endocrinol* 2014; 171: 527-533.

Address for correspondence

Agnieszka Adamczyk
 Department of Applied Radiobiology
 Maria Skłodowska-Curie Memorial
 Cancer Centre and Institute of Oncology,
 Cracow Branch
 ul. Garncarska 11
 31-115 Krakow, Poland
 tel. +48 12 634 83 71
 fax +48 12 423 10 76
 e-mail: aa.adamczyk@yahoo.com