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Prognostic value of HER3, PTEN and p-HER2 expression in patients with HER2positive breast cancer*

Wartość prognostyczna ekspresji HER3, PTEN i p-HER2 u chorych na HER2-dodatniego raka piersi

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Summary

Background HER2 overexpression is an unfavorable prognostic factor in patients with breast cancer, but it is also a target for the monoclonal antibody trastuzumab, which is effective in adjuvant and palliative settings. HER2 positivity is an inclusion criterion for immunotherapy, but it is not a positive predictive factor, and only half of patients benefit from the treatment.

Aim: The aim of this study was to evaluate the prognostic and predictive value of HER3, PTEN and phosphorylated HER2 (p-HER2) expression in primary breast tumors of patients treated with trastuzumab in an adjuvant or palliative regimen.

Material/Methods: Immunohistochemical (IHC) analysis with 3 antibodies specific to the proteins was performed in tumor specimens obtained from 81 HER2-positive patients treated with trastuzumab.

Results: HER3 overexpression was present in 55.6% of the examined tumors, and PTEN or pHER2 positivity was present in 32.0% and 34.6% of them, respectively. HER3 overexpression and PTEN positivity correlated with larger tumor size (p=0.016 and p=0.008, respectively). p-HER2 positivity correlated with more advanced clinical stage of the disease (p=0.032). There was no correlation between the proteins' expression and survival for 31 patients treated with trastuzumab in the palliative regimen.

Discussion: HER3 overexpression, PTEN positivity and p-HER2 positivity in tumor cells of HER2-positive patients correlate with more advanced clinical stage of breast cancer. Expression of these proteins does not predict outcome of trastuzumab treatment.

Keywords: **HER3 • PTEN • phosphorylated HER2 • HER2-positive breast cancer**

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Abbreviations: **AKT2** – serine/threonine kinase 2, **CISH** – chromogenic *in situ* hybridization, ***cMET*** – protooncogene that encodes a protein known as hepatocyte growth factor receptor (HGFR), **CR** – complete response, **DFS** – disease-free survival, **ER** – estrogen receptor, **FISH** – fluorescence *in situ* hybridization, **HER1-4** – human epidermal growth factor receptor 1-4, **IGF1R** – insulin-like growth factor 1 receptor, **MAPK** – mitogen-activated protein kinase, **OS** – overall survival, **OStrast** – overall survival from start of trastuzumab, **PD** – progressive disease, **PDK1** – phosphoinositide dependent kinase 1, **PDK2** – phosphoinositide dependent kinase 2, **PFS** – progression-free survival, **p-HER2** – phosphorylated HER2, **PIP2** – phosphatidylinositol-4,5-bisphosphate, **PIP3** – phosphatidylinositol-3,4,5-trisphosphate, **PI3K** – phosphatidylinositol 3-kinase, **PRg** – progesterone receptor, **PR** – partial response **PTEN** – phosphatase and tensin homolog, **Ras** – Rat sarcoma GTPase protein, **SD** – stable disease, ***TGFA*** – transforming growth factor alpha gene.

Background

HER2 is one of four members of the human epidermal growth factor receptor family (HER1 – HER4) [1,50,53]. These membrane receptors with tyrosine kinase activity are widespread in many different kinds of tissues and are involved in signal transduction crucial for normal development and growth [2]. HER receptors have similar structure and cooperate during signal transduction. They consist of [1,10,11,29,44,52]:

- an external domain, which is recognized by specific ligands (HER2 is an exception; its ligand has not been identified yet);
- a transmembrane domain;
- an internal domain composed of a part with tyrosine kinase activity (HER3 lacks this activity) and a C-terminal fragment, which is phosphorylated during activation.

HER receptors are activated by their ligands [44]. Activation evokes conformational changes, dimerization, initiation of tyrosine kinase activity and mutual phosphorylation of C-terminal fragments [10,15,29,33,44,52,62]. This last step is essential for signal transduction into the cell and accomplishing biological effects such as growth, proliferation, migration, arrest of apoptosis and release of angiogenic factors. Signal transduction from activated membrane receptors to the nucleus is facilitated by signal molecules chained into signal pathways. Different HER receptors cooperate with specific molecules.

The heterodimer formed of HER2 and HER3 is considered to have the greatest potential for signal transduction [62]. HER3 lacks tyrosine kinase activity, but it has the highest number of tyrosine residues in the C-terminal fragment, which after phosphorylation become docking sites for signal molecules. Phosphorylated HER3 cooperates with phosphoinositide 3 kinase (PI3K), which, once activated, transmits a signal to the Akt-dependent pathway. This phenomenon may be reversed by PTEN phosphatase activity. Figure 1 shows this mechanism.

Thirty years of research on HER2 function shows that HER2 overexpression is engaged in development of some neoplasms such as neural tumors, gastric cancer, ovarian or endometrial cancer [4,22,32,69]. Considerable knowledge about this phenomenon has been achieved in the field of breast cancer [1,45,52,58,63,65]. About 17-33% of breast cancers are HER2-positive, i.e. receptor overexpression can be detected in cancer cell membrane with immunohistochemistry or its gene amplification can by revealed with fluorescence or chromogenic in situ hybridization (FISH or CISH) [6,75]. HER2 positivity in patients with breast cancer is correlated with shortening of disease-free survival (DFS) and overall survival (OS) in operable disease [3,4,21,35,42,49,57,70]. The poorest prognosis characterizes patients with HER2 overexpression and lymph node involvement [19,45]. However, HER2 can also be a target for biological anti-cancer therapy [20]. The monoclonal antibody trastuzumab is the first medicament from this group of compounds, and nowadays it is a well-established part of adjuvant and palliative treatment [5,6,13,36,45,59,62,71]. Regrettably, only half of patients with metastatic HER2-positive breast cancer treated with trastuzumab achieve clinical benefit [5,59,71]. As primary and secondary immunoresistance is a significant problem in clinical practice, searching for predictive factors of anti-HER2 therapy is very important. There are many theories explaining lack

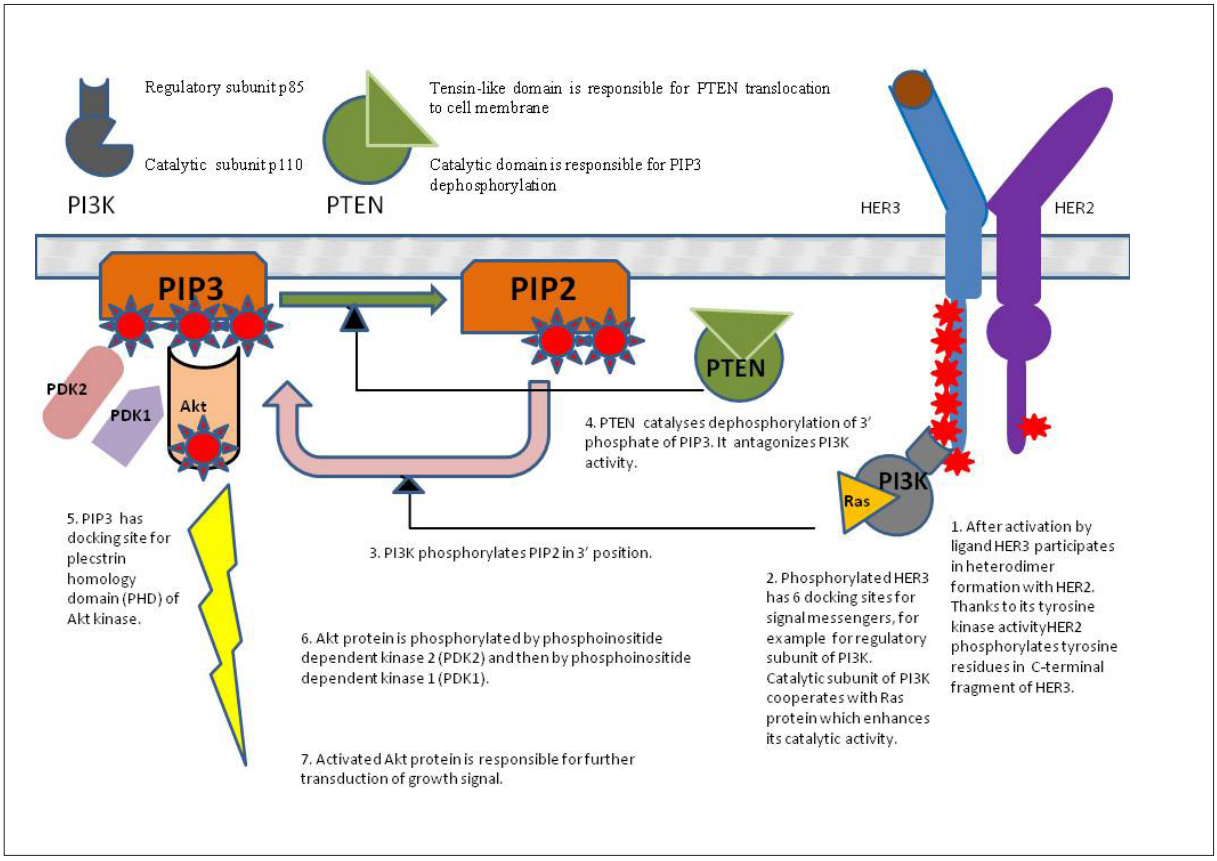


Fig. 1. The role of PTEN and PI3K proteins in transduction of growth signal from HER2/HER3 heterodimer

of trastuzumab activity, for example: deficiency in PTEN activity, HER3 overexpression or expression of phosphorylated HER2 [25,36,67,68].

PTEN is the main repressor of the signal transmitted by HER2/HER3 heterodimers [12,16]. Preclinical studies show that normal PTEN activity is essential for trastuzumab-dependent cessation of the growth signal [14,34,37]. Germinal mutations in this suppressor gene cause inherited disorders (PTEN hamartoma tumor syndromes), for example Cowden's syndrome or Bannayan-Riley-Ruvalcaba syndrome [16,23]. Females with Cowden's syndrome have 25-50% lifetime risk of developing bilateral multifocal breast cancer.

HER3 owing to its six phosphorylated tyrosine residues is an important growth signal enhancer [24,72]. Preclinical studies demonstrated that lack of HER3 may inhibit the oncogenic potential of HER2 [56]. On the other hand, HER3 can be relevant to trastuzumab resistance due to its possible cooperation with the insulin-like growth factor 1 receptor (IGF1R) or cMET receptor [24,25,31].

HER2 phosphorylation in the 1248 tyrosine residue (p-HER2) is a marker of high signaling activity [19,26]. There are data which indicate that phosphorylation in this residue confers oncogenic transformation due to its cooperation with the Ras/MAPK pathway. Some authors have observed that p-HER2 expression is accompanied by HER1 presence [18]. Preclinical studies revealed that transfection of cells with the genes *HER2* and transforming growth factor alpha (*TGFA*), a ligand for HER1, may induce aggressively developing cancers.

The aim of this study was to perform a retrospective assessment of the prognostic and predictive value of PTEN, HER3 and p-HER2 expression in tumor cells of patients with HER2-positive breast cancer, who underwent immunotherapy with trastuzumab.

Material and Methods

Eighty-one women with breast cancer treated with trastuzumab in 2003-2010 at the Department of Oncology, Copernicus Memorial Hospital in Łódź, Poland were eligible for the study. The study was approved by the Bioethics Committee at the Medical University of Łódź (approval no. RNN/138/09/KE). Depending on disease stage and clinical approach, patients were categorized into one of three groups:

I.45 women were treated with trastuzumab in an adjuvant regimen after primary surgery performed in 2006-2009. The follow-up interval from primary diagnosis to the date of death or the last observation varied between 13 and 57 months (median 37 months). During this period recurrence was observed in 8 patients. Two of them continued trastuzumab beyond progression with palliative chemo-

therapy, 3 patients were treated with lapatinib combined with capecitabine, 1 patient received palliative chemotherapy and 2 patients received palliative hormone therapy. Two patients died during this period.

II.31 women were treated with trastuzumab in a palliative regimen because of metastatic disease with or without chemotherapy in 2003-2010. All of them were primarily operated on and received adjuvant treatment without trastuzumab. The follow-up interval from primary diagnosis to the date of death or the last observation was between 19 and 139 months (median 62 months). Eight patients initially were treated with trastuzumab as monotherapy, and 3 of them continued immunotherapy beyond progression in combination with chemotherapy. Twenty-three patients were treated with trastuzumab combined with chemotherapy. Twelve of them continued trastuzumab beyond progression with a switch to a different chemotherapy. Patients received up to 5 lines of palliative chemotherapy. Four patients were treated with palliative hormonal manipulations without trastuzumab. Twenty deaths were observed in this group.

III.Five women had a different course of treatment than described above. Two patients were treated with trastuzumab in a preoperative regimen and then they under-

Table 1. Presents patient clinical characteristics.

Patient characteristics	Patients treated with trastuzumab in adjuvant regimen (n=45)	Patients treated with trastuzumab in palliative regimen (n=31)	Other patients (n=5)
Median age at the diagnosis	54.3	50.2	54.3
Preoperative chemotherapy			
Yes	14	15	3
No	31	16	2
Mastectomy	29	29	4
Breast conserving surgery	16	2	0
Adjuvant chemotherapy			
Yes	45	30	1
No	0	0	4
Adjuvant hormonal therapy			
Yes	20	11	0
No	25	20	0
Adjuvant radiotherapy			
Yes	36	19	2
No	9	12	3
T1-T2	38	19	2
T3-T4	7	12	3
N (-)	14	7	0
N (+)	31	14	5
Disease stage			
I-II	25	20	0
III	20	11	2
IV	0	0	3
Palliative therapy with trastuzumab			
Monotherapy	0	8	2
Combined with chemotherapy	2	23	3
Number of lines of palliative treatment			
1-2	6	16	1
3-5	0	15	4
Immunotherapy started as 1. line of palliative treatment			
Yes	2	19	0
No	0	12	5
Trastuzumab beyond progression			
Yes	2	12	2
No	43	19	3

went surgery. Three patients were diagnosed with primary metastatic disease and received trastuzumab in a palliative regimen. Three patients from this group died.

Primary breast tumors routinely fixed in formalin and embedded in paraffin were collected. They were immunohistochemically assayed for expression of PTEN, HER3 and p-HER2.

Monoclonal antibodies anti-PTEN clone 28H6 (Novocastra), anti-HER3 clone DAK-H3-IC (Dako) and anti-p-HER2-pY-1248 clone PN2A (Dako) were used in IHC analysis. Immunostaining was performed according to the manufacturer's instructions. PTEN expression was visualized with the Dako EnVision+ System, HER3- and p-HER2 with the CSA II Biotin-Free Catalyzed Amplification System. Expression of these proteins was identified by a pathologist with a light microscope.

PTEN protein displayed a nuclear staining pattern. Tonsil tissue provided a positive control. The intensity and distribution of the staining were scored according to the model shown in Table 2 [16]. In conformity with the scoring, patients were dichotomized into PTEN+ and PTEN- groups. Due to the shortage of samples the analysis comprised 78 patients.

Table 2.

Staining distribution / Staining intensity	Diffused >50% cancer cells +	Regional 50-15% cancer cells +	Focal <15% cancer cells +
Intense	PTEN +	PTEN +	PTEN +
Moderate	PTEN +	PTEN -	PTEN -
Weak	PTEN -	PTEN -	PTEN -

HER3 displayed a cytoplasmic staining pattern. Prostate epithelium provided a positive control. Staining score was calculated as [1 x % of cells with weak staining + 2 x % of cells with moderate staining + 3 x % of cells with strong staining] [1]. All scores were within the range 0-300. The median score (180) was assumed as a cut-off point between HER3 overexpression and its lack.

For anti-p-HER2 antibody there was membrane and cytoplasmic staining. According to other authors, cytoplasmic staining without membrane staining was considered negative [17,27,64]. Every membrane staining of cancer cells was recognized as positive, because even weak p-HER2 expression represents active status of the HER2 receptor [17,27].

Other collected data: hormonal receptor (HR) expression, grading, histological type, tumor size and nodal status were included in routine histological reports made for the patients in pathology departments of 3 hospitals in Łódź: the Copernicus Memorial Hospital, the Polish Mother Memorial Hospital, and the Department of Internal Affairs Hospital. Table 3 presents patients' histopathological characteristics.

For 30 patients, due to preoperative systemic treatment, primary staging of disease was based on clinically evaluated tumor size and nodal status. For the same reason, grading assessment was impossible for 17 patients. Before the start of trastuzumab treatment, HER2 status was assessed in all patients with the commercially available HercepTest (Dako). Patients with a score of 3+ were recognized as HER2-positive, those with 0 or 1+ as HER2-negative. In patients with a score of 2+, HER2 status was further validated with fluorescent in situ hybridization (FISH) – cases with an increased gene copy number were regarded as HER2-positive. Only HER2-positive patients were eligible for trastuzumab treatment and for this study.

Correlations between PTEN, HER3 and p-HER2 expression and other variables such as tumor size, nodal status, grading, hormonal receptor expression, age at the diagnosis, and staging were analyzed for all patients.

The associations of the protein expression and various survival endpoints as well as objective response to immunotherapy were also analyzed. This analysis comprised only 31 patients treated with trastuzumab in a palliative regimen (II group), because of the longest observation period and similarity of treatment courses. The survival endpoints included: disease-free survival (DFS), defined as the interval from primary operation to the relapse (prior to the start of any palliative treatment); progression-free survival (PFS), defined as the interval from the date of trastuzumab start to the date of first progression or death; overall survival (OS), defined as the interval from the date of diagnosis to the date of death or last observation; and overall survival from the start of trastuzumab (OStrast), defined as the interval from the start of trastuzumab to the date of death or last observation. Survival rates were estimated according to the Kaplan-Meier product limit method. Survival distributions were compared using the log-rank test.

Kruskal-Wallis analysis of variance and Fisher›s exact test were used to verify relations between the expression of the proteins and biological and clinical characteristics of the patients. Statistical significance was assumed when two-sided $p<0.05$. To analyze data and generate graphs, StatsDirect (StatsDirect Ltd., England) and Statistica 9.1 (StatSoft Inc.) software was used.

Results

Table 3 presents histological and immunohistochemical features of examined tumors.

Tumors obtained from patients treated with adjuvant trastuzumab were slightly more often positive for all studied proteins than patients treated for metastatic disease, but the difference did not reach statistical significance.

Associations between the expression of PTEN, HER3, p-HER2 and clinical or biological features are shown in

Table 3.

Patient histopathological characteristics	Patients treated with trastuzumab in adjuvant regimen (n=45)	Patients treated with trastuzumab in palliative regimen (n=31)	Other patients (n=5)	Total (N=81)
Histopathological type				
Ductal	43	27	5	75
Lobular	1	2	0	3
Apocrine	1	1	0	2
HercepTest 3+	40	28	4	72
HercepTest 2+/FISH+	5	3	1	9
G2	19	5	2	26
G3	19	16	3	38
Gx	7	10	0	17
ER/PR+	24	9	1	35
ER/PR-	21	22	4	46
HER3+	26	15	4	45 (55.5%)
HER3-	19	16	1	36 (44.5%)
PTEN+	14	8	3	25 (32.0%)
PTEN-	31	20	2	53 (68.0%)
p-HER2+	15	9	4	28 (34.7%)
p-HER2-	30	22	1	53 (65.3%)

Table 4. Patients with PTEN expression or HER3 overexpression were more likely to have bigger tumors (p=0.008 and p=0.016, respectively). An association between p-HER2 expression and more advanced TNM staging of the disease was also found (p=0.032). p-HER2+ patients showed a tendency to have lymph node involvement more frequently (p=0.082). There were no other associations between immunohistochemical and biological or clinical features.

The expression of PTEN, HER3 or p-HER2 had no prognostic effect on any studied survival endpoint in the group treated with palliative trastuzumab-based regimens.

Expression of these proteins also did not have any predictive value for response to palliative immunotherapy (Table 5).

Although there was no statistically significant difference in response to the treatment between patients who received a trastuzumab-based regimen as the first or subsequent line of therapy, an objective response was achieved by 12 of 31 patients treated with palliative trastuzumab, and nine of them received immunotherapy in the first line. No statistically significant association between the moment of trastuzumab start (first or subsequent line of treatment) and PFS was found, even though median values were surprisingly different (12.5 months v. 5.6 months, respectively; 8 months for all patients). The patients treated with trastuzumab as the first line of palliative treatment had an obvious tendency to live longer from the start of the immunotherapy than the patients treated with the antibody as a subsequent line of the therapy (median 24 months v. 10.5 months, p=0.057).

Discussion

In this study, in the majority of patients ductal type of breast cancer was observed. High-grade tumors, lack of expression of hormonal receptors and involvement of lymph nodes were also predominant. These data are concordant with other studies with HER2-positive breast cancer patients [4,7,24,35,40,49,61].

Diminished PTEN expression was observed in 68% of the patients eligible for this study. According to other authors, 33-48% of all breast cancers are associated with a decrease or lack of PTEN expression [16,46,47]. Similarly, 36-49% of HER2-positive breast cancers are PTEN--deficient [20,34]. The apparent difference between this result and other reports may be due to limitation of the immunohistochemical technique or the small number of patients.

Like in other studies, a nuclear pattern of staining for PTEN was observed in this study (Fig. 2A) [46,47,55,73]. However, cytoplasmic staining is also possible [16,20,46]. The scoring system proposed by Depowski et al. [16], taking into account both intensity and distribution of the staining in cancer cells, was used here. The same cut-off point was adopted as well.

We found an association of PTEN expression with larger size of the tumors, but not with other clinical or histopathological features. Similar findings were obtained by Perez-Tenorio et al. [46]. In their study, PTEN negativity was associated with smaller size of tumors (p=0.022), presence of PI3K mutation (p=0.002), ER expression (p=0.002), HER2 negativity (p=0.011) and lack of AKT2 expression (p=0.027). These results are opposed by those

Table 4.

Feature	HER3- (n=36)	HER3+ (n=45)	p	PTEN- (n=53)	PTEN+ (n=25)	p	p-HER2- (n=53)	p-HER2+ (n=28)	p
T1-2	31	28	0.016	43	13	0.008	39	20	0.836
T3-4	5	17		10	12		14	8	
N0	12	9	0.174	15	6	0.689	17	4	0.082
N+	24	36		38	19		36	24	
ER/PR+	16	19	0.841	23	11	0.960	23	12	0.963
ER/PR-	20	26		30	14		30	16	
G3	16	22		24	13		26	12	
G2	14	12	0.451	19	7	0.811	16	10	0.869
Gx	6	11		10	5		11	6	
Median age at the diagnosis	53	52	0.537	53	52	0.640	50.9	56.3	0.128
TNM									0.032
I-II	21	24	0.653	31	12	0.385	34	11	
III-IV	15	21		22	13		19	17	

Table 5.

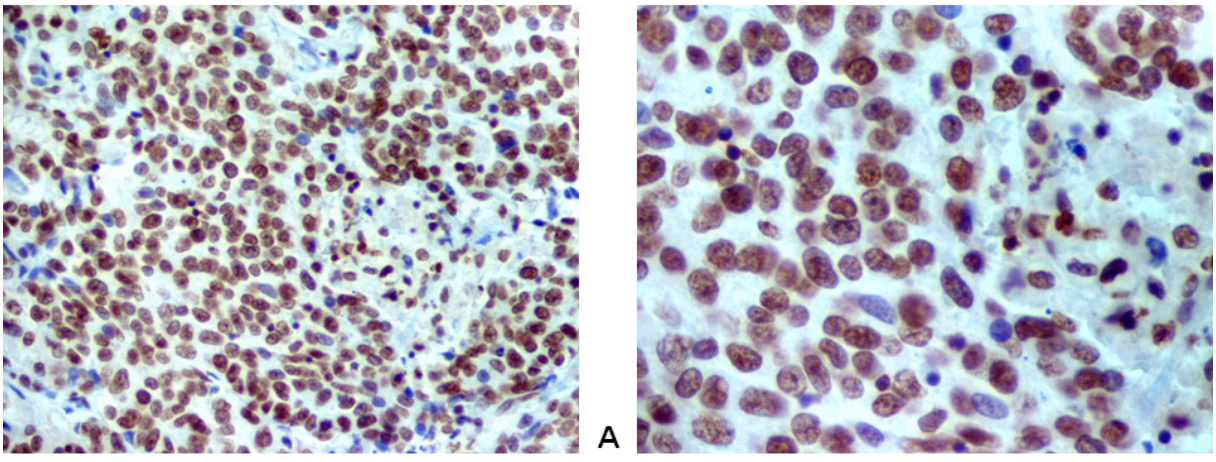
Objective response	HER3 overexpression			PTEN expression			p-HER2 expression		
	Her3- (n=16)	Her3+ (n=15)	p	PTEN- (n=20)	PTEN+ (n=8)	p	p-HER2- (n=22)	p-HER2+ (n=9)	p
PD+SD	8	11	0.183	14	4	0.400	14	5	0.704
PR+CR	8	4		6	4		8	4	

of Shi et al. [54] and Bose et al. [9], who observed that PTEN negativity was more frequent in HR-negative patients with breast cancer. Bose et al. found a tendency in PTEN-negative patients to have more advanced disease (p=0.091). Research by Perren et al. [47] confirmed the association of PTEN negativity with HR negativity. PTEN-positive patients eligible for their study had a tendency to have tumors of lower grading and smaller size, although the number of patients was small. According to Depowski et al. [16], the majority of PTEN-negative patients were ER-negative; lymph node involvement and death caused by cancer were observed among them more frequently. A study by Piekarski et al. [48] confirmed the association of PTEN negativity with lymph node metastases. Studies by Perez-Tenorio et al. [46] as well as Panigrahi et al. [41] failed to show an impact of PTEN status on disease-free survival.

The results of this study show an association of PTEN positivity with larger tumor size, which is recognized as a weak negative prognostic factor. Therefore it conflicts with most of the above-mentioned studies. It is noteworthy that those studies involved HER2-positive and negative patients, whereas only HER2-positive patients were eligible for our study.

We did not find any association of PTEN status and survival endpoints or response to trastuzumab treatment, although this second phenomenon was previously observed by other researchers. HER2-positive patients treated with palliative trastuzumab combined with taxane were eligible for studies by Nagata et al. [37] and Fujita et al. [20]. PTEN-positive patients were more likely to achieve a partial or complete response to the treatment (p<0.01 and p=0.003, respectively).

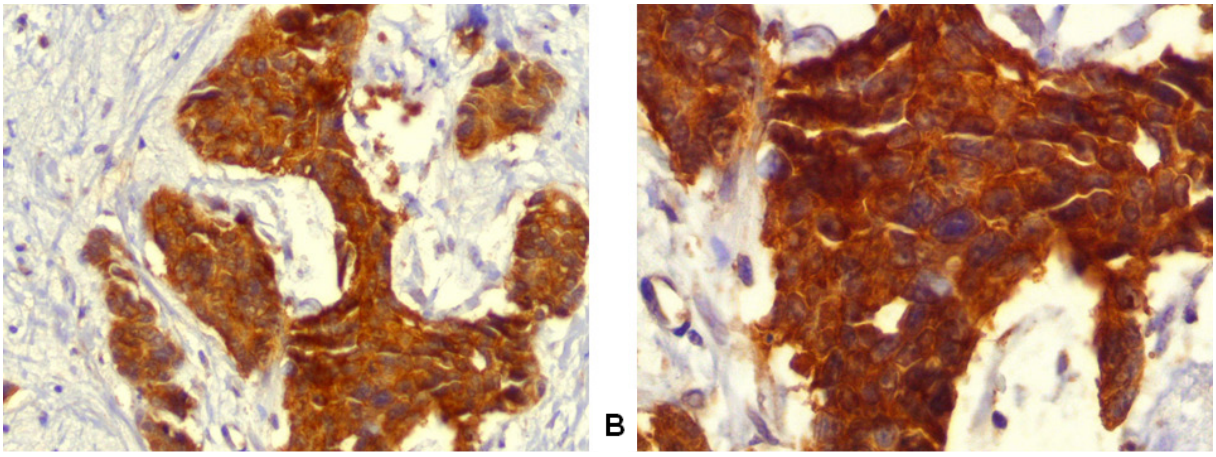
Assuming that the median value of HER3 scoring served as the cut-off point, HER3 overexpression was observed in 55% of the patients in this study. According to other researchers, overexpression of the receptor applies to 14-75% of all breast cancer types [27,49,51,66,67], although Travis et al. [66] claim that the feature is more frequently observed among patients with metastatic (35%) than operable disease (15%). On the other hand, El-Rehim et al. found HER3 overexpression in 50% of HER2-positive patients [1]. In the Naidu et al. study, 63% of HER2-positive patients were HER3-positive as well [38]. Such a discrepancy between these results may result from differences between study populations and various methods of research such as antibodies, scoring systems and, of course, cut-off points. The scoring system and the cut-off point in this study was adopted from El-Rehim et al. [1]. In accordance with other studies, the cytoplasmic staining pattern was observed [38,49,66], but cancer cell membrane could be stained as well (Fig. 2B) [30,51].



A

Immunohistochemical staining for PTEN – magnification 200x.

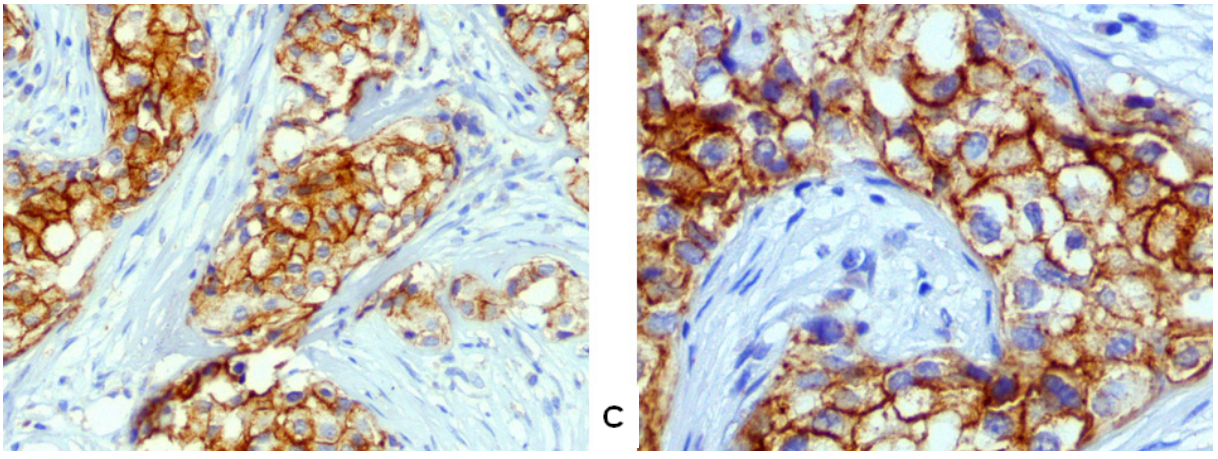
Immunohistochemical staining for PTEN – magnification 400x.



B

Immunohistochemical staining for HER3 – magnification 200x.

Immunohistochemical staining for HER3 – magnification 400x.



C

Immunohistochemical staining for p-HER2 – magnification 200x.

Immunohistochemical staining for p-HER2 – magnification 400x.

Figure 2. Immunohistochemical staining for PTEN, HER3 and p-HER2.

Association of HER3 overexpression with larger tumor size was found in our study. However, we did not observe any correlation between HER3 positivity and other features, survival or response to immunotherapy.

The association of HER3 overexpression with tumor size was also found by Travis et al. [66]. Furthermore, El-Rehim et al. [1] and Bieche et al. [8] observed that HER3-positive patients had involvement of lymph nodes more frequently than HER3-negative ones. There are some disputes about the putative prognostic value of HER3 overexpression. Some researchers have observed an association between the receptor's presence and higher grading of breast tumors [38,49] or shorter DFS [51,52] or time to local recurrence [66]. Others have found HER3-overexpressing patients to have positive prognostic features such as lower grading [27,30,43] and HR expression [27,28,30,43] more frequently, or longer DFS or OS [30,43]. However, it should be mentioned that both HER2-positive and HER2-negative patients were eligible for the quoted studies, and methods used by the researchers were different. Studies by Lee et al. [30] and El-Rehim et al. [1] clarified this controversy to a certain degree. The researchers showed that coexpression of HER3 with other HER receptors was associated with shorter DFS or OS. Thus simultaneous assessment of different HER receptors' expression in cancer cells may be a more valuable prognostic factor than assessment of expression of only one receptor.

There are some controversies in preclinical studies about the predictive value of HER3 overexpression for trastuzumab treatment [31,39]. Yonemori et al. [74] failed to find an association between the receptor's overexpression and response to trastuzumab-based preoperative systemic treatment in patients with HER2-positive breast cancer. The same applies to Smith et al. [60], who examined the impact of HER3 expression on response of patients to palliative immunotherapy. However, in this study the result might have been affected by the extremely large proportion of HER3-positive patients (70 out of 77 patients).

In our study, 35% of the examined tumors were immunopositive for p-HER2. Other authors have reported that this feature applied to 12-40% of patients with HER2-overexpressing breast cancers [1,17,18,26,27,64]. All quoted researchers used the same antibody as we did in our study. Cancer cells demonstrated membrane and cytoplasmic staining (Fig. 2C) [17,27]. According to previous studies, only the membrane stained tumors were considered as truly p-HER2-positive. The scoring values ranged from 10% to 95%. Considering the assumption made by DiGiovanna et al., even weak or non-extensive p-HER2 immunostaining may represent receptor activation [51]. Such staining was regarded as positive in our study as well.

In our study, p-HER2-positive patients were significantly more often diagnosed at stage III-IV of the disease (p=0.032), and they tended to have involvement of lymph nodes more frequently (p=0.082).

Thor et al. observed an association of p-HER2 expression with some negative prognostic factors such as younger age of disease onset, higher grading, bigger tumor size, and HR negativity [64]. Similar observations were made by DiGiovanna et al. [18], who also found some relation between p-HER2 positivity and presence of lymph node metastases as well as a greater number of involved lymph nodes. p-HER2-positive patients had shorter DFS (p=0.032) and tended to have shorter OS (p=0.078). Frogne et al. focused on another residue of HER2 phosphorylation – Y1221/1222 [19]. They found an association between presence of this phosphorylation in cancer cells and shorter DFS (p=0.001) and OS (p=0.009). However, patients eligible for the quoted studies had not been treated with trastuzumab.

Our study failed to demonstrate predictive value of p-HER2 expression for trastuzumab treatment. However, Hudelist et al. [26,27] reported that p-HER2-positive patients treated with trastuzumab had longer PFS (median 11.7 v. 4.5 months, p=0.001). Their further study also showed that p-HER2-positive patients tended to have a better response to trastuzumab (p=0.063) and longer PFS (median 7.5 v. 4.5 months, p=0.066). Furthermore, patients with p-HER2 and p-HER1 coexpression had significantly greater clinical benefit from the treatment (p=0.041) and longer PFS (median 25.8 v. 4.5 months, p=0.026).

Some disagreements between the results of the present research and data from the quoted studies may be caused by the small number of patients enrolled in our study. In particular, the group treated with palliative immunotherapy, which provided data for survival calculations, was not numerous. This might explain the disappointing lack of impact of the proteins' expression on survival, although they are associated with some well-established prognostic factors. It is noteworthy that survival endpoints were analyzed in a specific group of patients, who were selected depending on occurrence of recurrent disease. This might have been important for DFS calculations.

Secondly, it is also possible that the results could have been affected by limitations of the immunohistochemistry method. This technique, apart from unquestionable advantages, has some drawbacks such as required experience in staining interpretation, different specificity of antibodies, different experimental protocols, and deterioration of tissue sample quality during long storage. In relation to this latter supposition, we found some differences between the proteins' expression in tumors stored for 1-14 years ("palliative" group II) and 1-4 years ("adjuvant" group I). For all antigens there was greater frequency of positive staining in the "adjuvant" group I, although the difference was not statistically significant.

Finally, a certain reason for conflicting data from this and other studies, especially according to the survival results, may by the heterogeneity of therapeutic approaches in the palliative group of patients.

To conclude, HER3 overexpression, PTEN expression and p-HER2 expression in breast cancer cells are associated with more advanced stage of disease. Expression of these proteins does not have predictive value for trastuzumab-based treatment in patients with recurrent HER2-positive breast cancer, who were primarily operated on and received adjuvant treatment other than immunotherapy. Verification of putative prognostic and predictive values of the proteins' expression necessitates a study recruiting a greater number of patients, especially those treated with adjuvant trastuzumab.

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