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Background: HER2 consists of an extracellular domain (the target for trastuzumab), a transmembrane domain, and an intracellular domain, which is recognized by an antibody used in the immunohistochemical assessment of HER2 overexpression. The study consisted of 31 patients with metastatic breast cancer treated with trastuzumab.

The aim of the study was to evaluate retrospectively expression of extracellular and intracellular HER2 domains in primary breast cancers treated with surgery and to assess their correlation with biological features of tumours, disease-free survival, overall survival, as well as progression-free survival and survival since the start of trastuzumab treatment.

Material and methods: All these patients relapsed and were treated with trastuzumab because of metastatic disease – none of them received trastuzumab in an adjuvant setting. Two kinds of antibodies were used in immunohistochemical evaluation: against extracellular and intracellular domains of the HER2.

Results: A significant correlation between different patterns of HER2 domain overexpression and disease-free survival was found. Disease-free survival (the time from the primary operation to relapse – prior to the start of any palliative treatment) was the shortest in patients with overexpression of both domains compared to patients without overexpression of both domains and the group with intracellular domain overexpression combined with absence of extracellular domain overexpression (median 13.2 vs. 24.3 vs. 52.4 months, $p = 0.01$). There was no significant difference between these 3 groups of patients in aspects of biological features of tumours, overall survival, progression-free survival and survival since the start of trastuzumab treatment.

Conclusion: Concurrent assessment of extracellular and intracellular domains of HER2 may have prognostic value for HER2-positive patients.

Key words: extracellular domain, intracellular domain, HER2, trastuzumab.

Prognostic value of expression of intracellular and extracellular domains of HER2 in patients with HER2-possitive breast cancer

Sylwia Dębska[1], Renata Kusińska[2], Urszula Czernek[1], Katarzyna Szydłowska-Pazera[1], Maria Błasińska-Morawiec[3], Izabela Dowgier-Witczak[3], Elżbieta Olas[3], Andrzej Kulig[4], Magdalena Jakubiak-Wielganowicz[5], Piotr Potemski[1]

[1]Department of Oncology, Medical University of Lodz
[2]Department of Pathology, Chair of Oncology, Medical University of Lodz
[3]Mikolaj Kopernik Voivodeship Specialist Hospital, Lodz
[4]Department of Clinical Pathomorphology, Polish Mother's Memorial Hospital – Research Institute, Lodz
[5]Department of Histopathology, MSWiA Hospital, Lodz

Introduction

25% of breast cancer patients overexpress HER2 [1-5]. The extracellular domain of the receptor is present on the cancer cell surface and it is a target for the anti-HER2 antibody trastuzumab. Nowadays immunotherapy with trastuzumab presents a part of adjuvant and palliative treatment of patients with HER2-positive breast cancer. Adjuvant treatment with the antibody allows for 38% reduction of relative risk of recurrence and 34% reduction of relative risk of death [6-10]. More than half of patients with metastatic breast cancer treated with trastuzumab-based regimens may achieve an objective response and they have longer progression-free survival and overall survival than patients treated with chemotherapy alone [11, 12]. Treatment with the antibody is slightly less effective as monotherapy – for this regimen the objective response rate is 19-26% [13, 14].

HER2-positive breast cancer is regarded as an especially aggressive subtype of the disease. It is associated with early relapse and some poor prognostic factors such as lymph node involvement, hormone receptor (HR) negativity, higher mitotic index and higher grading [2, 4, 5, 15, 16].

Only patients with HER2 overexpression are eligible for trastuzumab treatment. For this reason immunohistochemical assessment of the receptor expression in cancer cells in all newly diagnosed patients is obligatory. HercepTest is an immunohistochemical kit commonly used in Poland for HER2 testing. It includes rabbit anti-human HER2 protein which is specific for a fragment of the intracellular domain of the receptor. A dedicated scoring system is provided for the staining assessment – tumours with score 3+ are categorized as HER2-positive, 0 and 1+ tumours as HER2-negative. Tumours with intermediate score 2+ need validation with fluorescent in situ hybridization (FISH), which detects *HER2* gene amplification – only cases with an increased gene copy number are regarded as HER2 positive [17, 18].

HER2 is a 185 kDa phosphoprotein composed of 3 domains: extracellular, transmembrane and intracellular. The extracellular domain comprises a fragment recognized by trastuzumab. The intracellular domain has intrinsic tyrosine kinase activity [19, 20]. There was identified a shorter variant of HER2 known as p95 receptor due to its molecular weight [21].

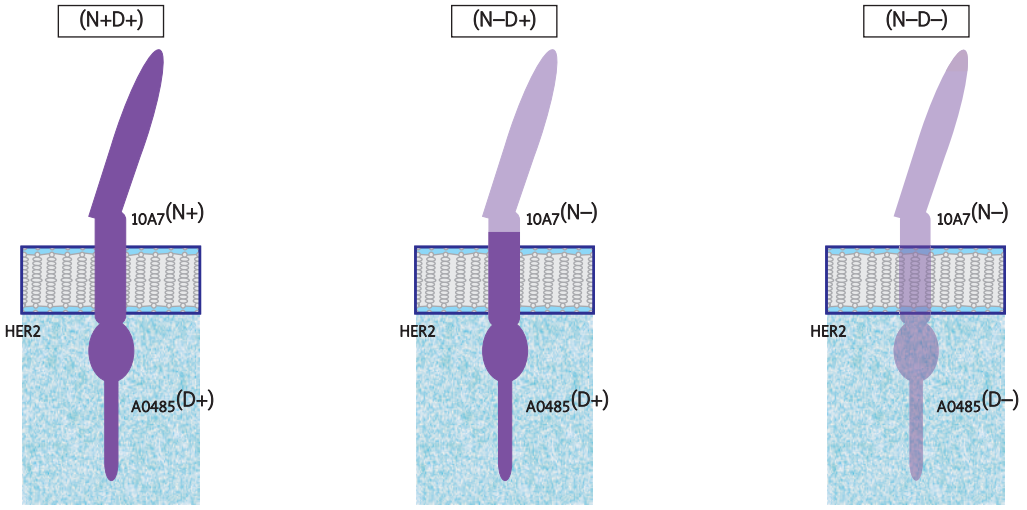

Fig. 1. Subgroups of patients dependent on coexpression of HER2 domains

Aim of the study

The aim of this study was to perform a retrospective analysis of extracellular and intracellular HER2 domains' expression in primary breast tumours of 31 patients treated with trastuzumab in a palliative regimen as well as assessment of its prognostic value and predictive value for immunotherapy.

Material and methods

Thirty one women with breast cancer treated with trastuzumab in 2003-2010 at the Oncology Department of Copernicus Memorial Hospital in Łódź, Poland were eligible for the study. All the patients were treated with trastuzumab in a palliative regimen with or without chemotherapy. All of them were primarily operated on and received adjuvant treatment without trastuzumab. The follow-up period from primary diagnosis to the date of death or the last observation varied between 19 and 139 months (median 62 mo.). Twenty-three patients were treated with trastuzumab combined with chemotherapy (CMF – 1, docetaxel – 12, etoposide – 1, paclitaxel plus carboplatin – 1, NF1 – 5, vinorelbine – 3). Four patients were also treated with palliative ET. Twenty deaths were observed in this group.

Patients' primary breast tumours routinely fixed in formalin and embedded in paraffin were collected. They were immunohistochemically assayed for expression of extracellular and intracellular domains of HER2.

Polyclonal rabbit anti-human c-erbB-2 oncoprotein (A 0485, Dako) against intracellular domain and mouse monoclonal antibody RTU-CBE-356 (10A7, Novocastra) against extracellular domain were used in immunohistochemical analysis. Immunostaining was performed according to the manufacturer's instructions and it was visualized with Dako En Vision TM+ System. The expression of domains was identified by a pathologist with a light microscope and assessed according to the HercepTest scoring system. For both antibodies there was membrane staining in cancer cells. For the purpose of this study only tumours scored as 3+ were regarded as positive ones; tumours scored as 2+, 1+, 0 were regarded as lacking the domain expression. Depending on co-expression of domains, patients were categorized as belonging to one of three groups presented in Fig. 1.

Other collected data – hormonal receptor expression, grading, histological type, tumour size and nodal status – were included in routine histological reports made for the patients in the pathology departments of 3 hospitals in Łódź: Copernicus Memorial Hospital, Polish Mother Memorial Hospital and Department of Internal Affairs Hospital.

For 13 patients, due to preoperative systemic treatment, primary staging of disease was based on clinically evaluated characteristics such as tumour size and nodal status.

The association between co-expression of HER2 domains and various survival endpoints as well as objective response to immunotherapy were analysed. These survival endpoints included the following: disease-free survival (DFS), defined as the time from the primary operation to relapse (prior to the start of any palliative treatment); progression-free survival (PFS), defined as the time from date of trastuzumab start to date of first progression or death; overall survival (OS), defined as the time from date of diagnosis to date of death or last observation; and overall survival from start of trastuzumab (OStrast), defined as the time from start of trastuzumab to date of death or last observation. Survival rates were estimated according to the Kaplan-Meier product limit method. Survival distributions were compared with the log-rank test.

Kruskal-Wallis analysis of variance and Fisher's exact test were used to verify correlations between the expression of both domains and biological and clinical features of the patients. Statistical significance was assumed when $p < 0.005$. To analyse data and generate graphs StatsDirect (StatsDirect Ltd., England) and Statistica 9.1 (StatSoft Inc.) software were used.

Results

Patient clinical and histopathological characteristics are presented in Table 1. All patients had been treated with radical surgery before relapse occurred. Most of the patients had

Table 1. Patient characteristics – stage of the disease, biological features of tumours, treatment

Patient characteristics	N+D+ (n = 11)	N–D+ (n = 10)	N–D– (n = 10)	p
Age at the moment of diagnosis – median	51	53.5	48	0.357
Neoadjuvant chemotherapy				0.235
Yes	6	5	4	
No	5	5	6	
Mastectomy	11	9	9	0.527
Breast conserving surgery	0	1	1	
Adjuvant chemotherapy				0.645
Yes	11	10	9	
No	0	0	1	
Adjuvant hormone therapy				0.999
Yes	4	4	3	
No	7	6	7	
Adjuvant radiotherapy				0.732
Yes	7	5	7	
No	4	5	5	
T1-T2	6	7	6	0.897
T3-T4	5	3	4	
N (–)	2	4	1	0.323
N (+)	9	6	9	
G1-2	2	2	1	0.756
G3	5	4	7	
Gx	4	4	2	
ER/PR+	3	3	3	0.999
ER/PR–	8	7	7	
TNM staging				0.521
I-II	6	8	6	
III	5	2	4	
Palliative trastuzumab started as				0.357
Monotherapy	3	4	1	
Combined with chemotherapy	8	6	9	
Number of lines of palliative regimens				0.172
1-2	8	5	3	
3-5	3	5	7	
Trastuzumab started as				0.156
1st line	9	4	6	
2nd – 5th line	2	6	4	
Trastuzumab beyond progression				0.732
Yes	4	3	5	
No	7	7	5	
Brain metastases				0.181
Yes	1	4	4	
No	10	6	6	

ductal breast cancer. One patient was diagnosed with lobular cancer and one patient with apocrine cancer. For 9 patients treated with preoperative chemotherapy, tumour grading was not possible. In 19 patients metastatic disease was localized in bones, soft tissue or lymph nodes. In 12 patients recurrence was observed in lungs, liver or brain.

All patients were screened for HER2 overexpression with immunohistochemical HercepTest.

Twenty-eight patients were scored as HER2 3+. Three patients were scored as HER 2+ and these results were further validated with FISH. In 22 patients results of HercepTest were concordant with immunohistochemical staining with antibody against the internal domain (D), but only in 11 patients they were concordant with the staining against the external domain (N).

The subgroup of patients with expression of both domains (N+D+) had the shortest disease-free survival compared with

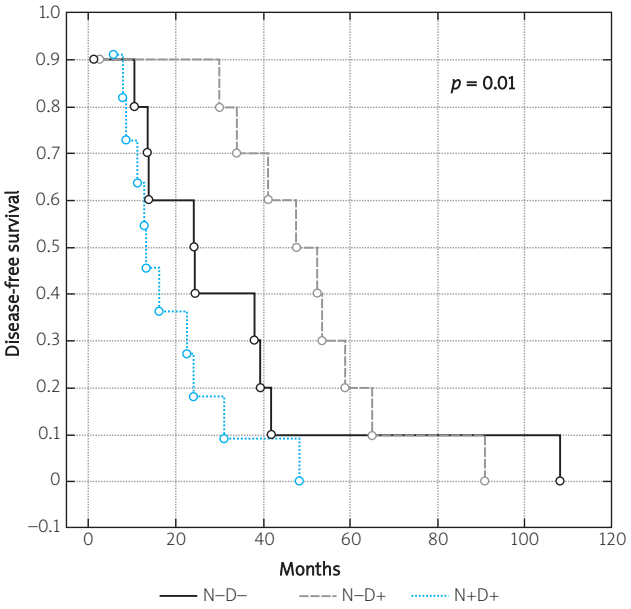

Fig. 2. Disease-free survival for patients with different expression of HER2 domains

the two other subgroups – the median DFS was 13.2 months in comparison with 24.3 and 52.4 months for N–D– and N–D+ subgroups respectively (p = 0.01) (Fig. 2). There was no association between the patterns of co-expression of domains and overall survival, progression-free survival or overall survival measured from the trastuzumab start date (Fig. 3).

In N–D– and N–D+ subgroups there were more patients who developed brain metastases during anticancer treatment than in the N+D+ subgroup (4/10 vs. 4/10 vs. 1/11), but it was not statistically significant. There was also neither a connection between patterns of expression of domains and other clinical and biological features nor a predictive value of their expression for the response to trastuzumab-based therapy.

There were some differences in response to the treatment between patients who received the trastuzumab-based regimen as the first or subsequent lines of therapy, but they were not statistically significant. 12 of 31 patients achieved an objective response, but 9 of them received immunotherapy in the first line. PFS median values for patients who were treated with trastuzumab as the first or subsequent line were quite different (12.5 vs. 5.6 months, respectively), but not significantly. Patients in the "first line" group also had a tendency to live longer from the start of the immunotherapy than the patients in the "subsequent line" group (median 24 vs. 10.5 months, p = 0.057).

Discussion

The majority of patients eligible for this study were diagnosed with ductal breast cancer; they had high grade tumours that lacked hormonal receptors and usually had lymph node metastases. These data are in agreement with other studies with HER2-positive breast cancer patients.

The results of the study indicate that the pattern of expression of HER2 domains may have additional prognostic value for patients with breast cancer. In patients who were primarily treated with breast surgery and adjuvant therapy without trastuzumab and then received the antibody due to recurrent, metastatic disease, different patterns of HER2 domains were associated with different disease-free survival. The patients with lack of external domain overexpression but with concurrent internal domain overexpression had the longest disease-free survival, while the patients with overexpression of both domains had the shortest. This is the first report concerning stratification of recurrence risk dependent on HER2 domains' co-overexpression in such a group of breast cancer patients.

However, only very specific patients were eligible for the study – patients who had the recurrence after radical surgery. Still there are no data about unselected patients with or without recurrent breast cancer, data that could be truly of prognostic value. The second important limitation of our study is the relatively small number of patients. It also should be mentioned that data from our study are not applicable to contemporary patients with operable HER2-positive breast cancer, because they are treated with adjuvant trastuzumab, which reduces the risk of recurrence [22-24]. So it could be interesting to investigate the prognostic and predictive value of different patterns of expression HER2 domains in patients treated with adjuvant trastuzumab.

But still these data suggest that unidentified biological mechanisms may be responsible for the disease course in patients with different patterns of expression of HER2 domains, and exploration of this mechanisms could broaden our knowledge about breast cancer.

Some differences between immunostaining patterns in this study and results of the HercepTest may be caused by limitations of the immunohistochemical method. Apart from unquestionable advantages such as quick results, easiness and convenience of implementation, availability in most laboratories, low cost and application for paraffin-fixed tissue, it has some significant drawbacks. It is not a quantitative assessment and it can be affected by various factors such as subjective staining interpretation, the need for collaboration with an experienced pathologist, diverse sensitivity and specificity of antibodies, diverse protocols of method, long time of storage of paraffin-embedded tissue, and heterogeneity of examined tumours.

HercepTest was approved by the FDA in 1998 and became a commonly used method in clinical practice. But there are various anti-HER2 antibodies which have different sensitivity and specificity. Table 2 presents some studies in which they were used.

Some researchers suspect that differences between expression of internal and external domains of HER2 may be due to the receptor engagement in formation of heterodimers [26]. Other authors suggest p95 expression as an explanation of this phenomenon. Some data indicate that p95 originates from extracellular domain shedding of HER2. If the extracellular domain is cleaved by a metalloproteinase, the intracellular domain at the inner surface of a cell membrane should remain [31, 32]. According to other observations, p95 is generated by alternative translation, which is epigenetically regulated. It is possible thanks to internal ribosome entry sites

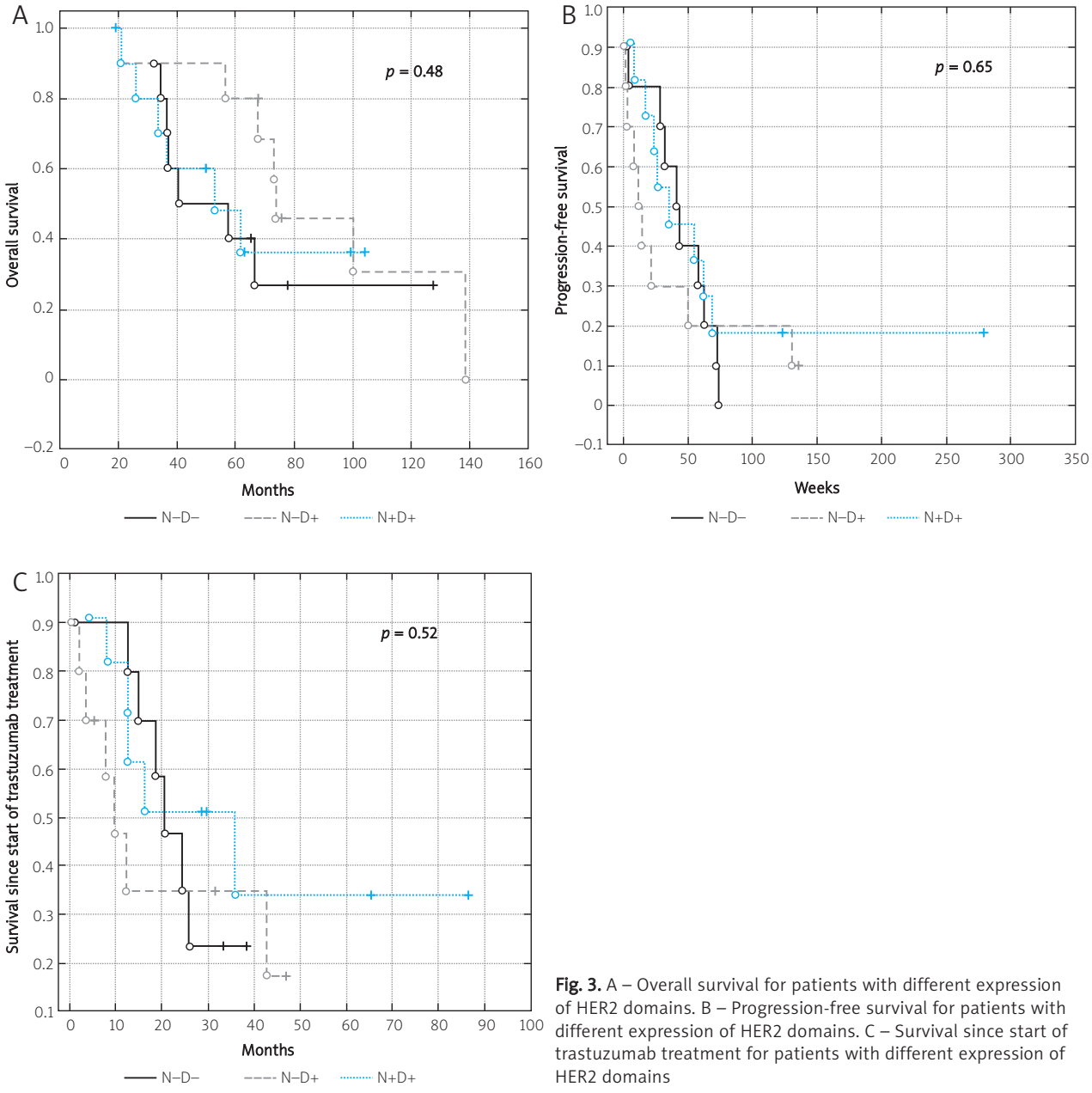

Fig. 3. A – Overall survival for patients with different expression of HER2 domains. B – Progression-free survival for patients with different expression of HER2 domains. C – Survival since start of trastuzumab treatment for patients with different expression of HER2 domains

(IRES) present in the *HER2* transcript as codons for methionine 611 and 687. These alternative protein products are also known as carboxyl-terminal fragments (CTF) [31-34].

P95 expression is regarded as a marker of poor prognosis and a negative predictive factor for trastuzumab treatment in patients with HER2-positive breast cancer [34]. Some studies have shown that 20-60% of HER2-positive tumours express p95 [34-36]. This wide range of occurrence is an implication of various research methods such as Western blot [34-36], immunofluorescence [34] or immunohistochemistry [37]. According to Saez *et al*., patients with higher expression of p95 in cancer cells measured with Western blot had shorter disease-free survival than patients with lower expression (median 32 vs. 139 months, $p < 0.0001$) [35]. P95 expression was associated with involvement of a higher number of lymph nodes ($p = 0.035$), and oestrogen and progesterone receptor negativity ($p = 0.013$ and $p = 0.001$, respectively). In patients treated with a palliative regimen based on trastuzumab, expression of p95 was associated with shorter progression-free survival and overall survival. Scaltriti et al. used an immunohistochemical method and found p95 expression in 9 of 46 patients with metastatic breast cancer treated with trastuzumab. Only 1 p95-positive patient had an objective response to the treatment, while 19 of 37 p95-negative patients responded to trastuzumab [34]. Similar patients were eligible for the Sperinde *et al*. study. They also found that patients with immunohistochemically detected expression of p95 had shorter progression-free survival (median 7.2 vs. 12.6 months, $p = 0.007$) and overall survival (median 29 vs. 48 months, $p = 0.012$). P95-positive patients also

Table 2. Examples of studies evaluating HER2 expression with immunohistochemical tests using different antibodies

Author	Anti-HER2 antibody	Study characteristics	Results
Ceccarelli (1999) [26]	anti-internal domain antibody – clone CB11, anti-external domain antibodies – clones CBE1 and Tab250	assessment of HER2 expression in unselected patients with breast cancer and its association with other biological features of primary breast tumours	co-overexpression of both HER2 domains was found in 26.5% of patients; internal domain overexpression without external domain overexpression was present in 10.8% of patients – they had involvement of axillary lymph nodes more frequently and more lymph nodes were involved in these patients
Ainsworth (2005) [27]	anti-external domain antibody CBE356, HerceptTest®	comparison of immunohistochemically assessed overexpression of HER2 and its gene amplification, assessment of their prognostic value in unselected patients with breast cancer treated with surgery	89% of patients with *HER2* amplification (FISH) had 2+ or 3+ score with CBE356 antibody, but 66% of them had 2+ or 3+ score with HerceptTest; patients with 2+ or 3+ score with CBE356 or HerceptTest® had shorter overall survival
Bussolati (2005) [28] Sapino (2007) [29]	biotinylated trastuzumab (biotHER)	assessment of HER2 expression in patients with breast cancer treated with palliative trastuzumab	27 of 54 patients had expression of HER2 external domain (Busolati) 47% of patients had expression of HER2 external domain (111 of 234) (Sapino) in both studies expression of external domain was associated with objective response to trastuzumab treatment and reduction of risk of progression and death
Vinhas-Ricardo (2007) [30]	anti-external domain antibody SP3, anti-internal domain antibody – clone CB11	assessment of HER2 expression in unselected patients with breast cancer (n = 119)	internal domain overexpression was present in 27 patients (CB11 3+), 22 of them also had external domain overexpression (SP3 3+)

had metastases to lung (64% vs. 32%, p = 0.015), brain (13% vs. 3%, p = 0.11) and skin (29% vs. 16%, p = 0.23) more frequently [37].

We assumed that expression of p95 could be detected by a specific antibody against its internal domain while the external domain was absent – N–D+ should be recognized as equivalent to p95 expression. In that case this research would oppose the above quoted studies. N–D+ patients had the longest disease-free survival (pure prognostic value) and there was no association between patterns of the expression of domains and biological features or response to trastuzumab (no predictive value). But this is only an assumption as we did not directly assess p95. Thus, the comparison between presented results and results of other studies seems to be quite difficult. Of note, there is no standard method of assessment of p95 expression. Furthermore, in our study patients were dichotomized due to the presence or lack of domain overexpression. Another drawback which could influence these results is the use of different antibodies and different scoring systems in the studies. In our study, the scoring system of HercepTest was used for both antibodies to enable uniform assessment. A 0485 and RTU-CBE-356 are examples of numerous anti-HER2 antibodies. Unfortunately their epitopes are unknown, while it is known that trastuzumab recognizes the fragment of extracellular domain near the cell membrane. It could be possible that RTU-CBE-356 (N) recognizes the epitope located above this point and there is a negative immunohistochemical reaction only when this fragment is lacking while the rest of the extracellular domain remains. That could explain why N(–) patients responded to trastuzumab. Based on this assumption, it can be suggested that p95 may not be the only truncated form of HER2.

N–D+ patients who had longer DFS did not have prolonged overall survival. They had a similar response to trastuzumab as other groups, so this does not explain such inconsistency between DFS and OS. Of course, this may result from the small number of patients eligible for the study and very diverse approaches to palliative treatment. Heterogeneity of trastuzumab-based treatment could disguise supposed predictive value of coexpression of HER2 domains.

In summary, we have found that simultaneous assessment of internal and external HER2 domains may have additional prognostic value for patients with HER2-positive breast cancer. Patients with HER2-positive breast cancer not treated with adjuvant trastuzumab who are positive for both external and internal domains of HER2 had the shortest disease-free survival in comparison with patients with other patterns of expression of domains. Thus, different patterns of expression of HER2 domains may reflect different courses of disease. However, a study including patients treated with adjuvant trastuzumab is needed to validate the expected prognostic value of co-expression of HER2 domains.

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Address for correspondence

Sylwia Dębska
Department of Oncology
Medical University of Lodz
Pomorska 251
92-213 Łódź
e-mail: sylwia.debska@o2.pl