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ORIGINAL ARTICLE

The role of p95HER2 in trastuzumab resistance in breast cancer

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Summary

Purpose: *Trastuzumab, the HER2 oncogene targeting drug, shows remarkable clinical efficacy in HER2-amplified breast cancer patients. Despite of robust activity, some of the patients with HER2-positive breast cancers do not get the benefit due to trastuzumab resistance. Overexpression of p95HER2 is one of the molecular mechanisms of trastuzumab resistance. The purpose of this study was to investigate whether p95HER2 overexpressing breast cancers were resistant to trastuzumab.*

Methods: *p95HER2 (truncated HER2) and HER2 were determined by real-time polymerase chain reaction (RT-PCR) analysis. HER2 protein expression and HER2 gene amplification were also determined by immunohistochemistry (IHC) and chromogenic in situ hybridization (CISH). Archival material from 80 formalin-fixed paraffin-embedded (FFPE) breast cancer tumor tissues was used for the study. None of the cases had metastases at the initial diagnosis. HER2-positive cases were treated with trastuzumab with/without chemotherapy.*

Results: *Of 80 breast cancer cases 39 (48.7%) were HER2-positive and had trastuzumab treatment. Of these 39 cases 11 (28.2%) were trastuzumab-resistant and 28 (71.8%) were not, 17 (43.6%) were recurrent cases and 22 (56.4%) were not. Three patients died during follow-up. p95HER2 mean ratio was 11.01±19.73 in 11 cases which were trastuzumab-resistant, while p95HER2 mean ratio was 1.99±1.37 in 28 cases without trastuzumab resistance. If p95HER2 ratio was low, there was no trastuzumab resistance. However, when p95HER2 ratio was high, there was trastuzumab resistance (p=0.210, Mann-Whitney U test).*

Conclusion: *p95HER2 was correlated with trastuzumab resistance, but it was not an independent factor of trastuzumab resistance. We claim that p95HER2 is sensitive but not specific for the prediction of trastuzumab resistance.*

Key words: *breast cancer, p95HER2, trastuzumab resistance*

Introduction

Breast cancer is one of the most common cancer types seen in female patients and is among the most common reasons of cancer-related deaths. It is a highly heterogeneous disease which can be microscopically divided into subgroups according to its morphologic structure and also into molecular subgroups by evaluating some specific biomarkers. Estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) are used for molecular classification and have a particular importance. HER2 overexpression and/or amplification is seen in approximately 20% of invasive breast carcinomas [1]. HER2 positivity is reported to be correlated with an aggressive clinical course and low survival rates [1-3]. Trastuzumab is a monoclonal antibody developed to act against the extracellular domain of HER2. It is an important targeted agent for both early and advanced-stage breast cancers. The course of HER2-positive breast cancer patients has changed since trastuzumab came

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into clinical use. Despite its stong efficiency, progression occurs in more than half of HER2-positive breast cancer patients, within one year or later, due to primary or acquired resistance [4,5].

Approximately in 30% of HER2-positive breast cancer patients, 90-115 kDa weighing receptor fragments all together known as p95HER2 carboxy terminal fragments (CTF) are expressed [6,7]. These p95HER2-CTFs are generated by two different mechanisms: proteolytic separation and alternative translation. Compared to tumors expressing HER2 receptor at full length, tumors expressing p95HER2 have poorer prognosis, have higher risk of metastasis and are resistant to trastuzumab treatment [8,9]. Most p95HER2/CTFs are inactive. However, only one of these CTFs (611-CTF) is an oncogenic form. This form is generated by alternative initiation of translation from the AUG codon at position 611 (611-CTF) and drives breast cancer progression in vivo [10]. Therefore, it is understood that the state of 611-CTF gene possibly influences the progression of HER2-positive tumors and using trastuzumab does not benefit such patients. For this reason, evaluation of either p95HER2 or p95HER2 with HER2 may be beneficial before the initiation of trastuzumab when planning treatment of HER2-positive breast cancer patients.

In this study, we aimed to investigate the predictive and prognostic roles of p95HER2 expression in trastuzumab resistance during breast cancer treatment.

Methods

One hundred and twelve patients diagnosed with invasive breast cancer during 2008-2012 were included in the study, which was approved by the Ethics Committee. Archived FFPE tissue blocks from 80 patients with primary invasive breast cancer were studied. Patient demographic data were obtained from electronic databased records and from relevant clinical doctors. Archived ready H&E preparations were attentively examined. Histological types (according to WHO 2012) and grades (in accordance with Nottingham grading system) of the tumors were determined. Presence of vascular and lymphatic invasion was evaluated. Tumors were classified with the AJCC-American Joint Committee on Cancer classification system according to the findings present at the time of initial diagnosis [11]. Tumors were classified as follows: T1 if tumor ≤2cm, T2 if 2-5cm, and T3 if tumor >5cm. Presence of chest wall invasion and/or direct invasion to skin (ulcerations and skin nodules) were classified as T4 no matter the size of tumor itself. Absense of metastasis to regional lymph nodes was classified as N0, metastases in 1-3 axillary lymph nodes as N1, while 4-9 metastases as N2. Ten and more metastatic axillary nodes were classified as N3. Metastatic lymph nodes were also examined to check for perinodal invasion.

Immunohistochemical investigations were completed at the time of first primary breast cancer diagnosis of the patients. Archived immunohistochemical material of ER, PgR, p53 and Ki67 was re-examined. ER and PgR and Ki67 were accepted as positive if the positively stained cells were ≥10%, ≥10%, and ≥14%, respectively. HIC staining was scored and categorized as negative (0 or 1+), equivocal (2+) or positive (+3) according to the ASCO/CAP guideline recommendations for HER2 testing in tumor tissue samples at the time of first diagnosis. If the results of HIC staining of HER2 were equivocal, in situ hybridization (ISH) as a reflex testing was performed. HER2 protein expression and HER2 gene amplification were assessed by re-dyeing the tissue samples. Additionally, HER2 and p95HER2 expressions were quantitatively assessed by RT-PCR. For this assessment, blocks containing living tumor tissue which were devoid of necrosis were used. The tumor region was identified in H&E sections of these blocks, a sample of tumor tissue was taken into Eppendorf tube and kept at room temperature until performing RT-PCR.

Because the primary goal of this study was to find out the role of p95HER2 in trastuzumab resistance, initiation and termination dates of trastuzumab were recorded. Date of local recurrence or metastasis, last date of follow up and last patient status were recorded.

Definition of trastuzumab resistance

There is not a common definition for trastuzumab resistance in the literature, and different definitions are often used interchangeably [12]. In this study, trastuzumab resistance was defined as the presence of progression detected in the first radiologic evaluation (performed at 8-12 weeks) of metastatic HER2 positive breast cancer patients who received first line therapy (trastuzumab±chemotherapy). Additionally, recurrences during adjuvant trastuzumab treatment within 12 months of treatment termination were defined as trastuzumab resistance [12]. Because HER2 positivity in breast cancer is known to be an independent factor for the development of brain metastasis, evaluation of trastuzumab resistance requires to consider also brain metastases [13]. If brain metastases were the first and single region of progression after trastuzumab treatment, this situation was not considered as trastuzumab resistance due to the high molecular weight of trastuzumab, leading to poorer penetration to central nervous system [13].

Statistics

Statistical evaluation was performed by using the SPSS16.0 software (SPSS Inc., Chicago, Ill). HER2 protein expression was evaluated with IHC and am-

Table 1. Clinicopathological chararacteristics of all cases in this study

Chararacteristics	*N (%)*
Age (years), mean±SD	52.8±12.5
Tumor size (cm), mean±SD	2.4±1.1
Localization	
Right breast	41 (51.2)
Left breast	39 (48.8)
Histologic type	
Invasive ductal carcinoma	70 (87.5)
Invasive lobular carcinoma	3 (3.8)
Other subtypes	7 (8.8)
Histologic grade	
I	3 (3.8)
II	54 (67.5)
III	23 (28.8)
Vascular invasion	
Yes	15 (18.8)
No	65 (81.2)
Lymphatic invasion	
Yes	49 (61.2)
No	31 (38.8)
Local recurrence	
Yes	2 (2.5)
No	78 (97.5)
Metastasis	
Yes	21 (26.2)
No	59 (73.8)
Stage	
I	18 (22.5)
II	37 (46.2)
III	25 (31.2)
IV	-

SD: standard deviation

Table 2. Biomarkers in primary tumor

Biomarkers	*N (%)*
ER	
Positive	55 (68.6)
Negative	25 (31.2)
PgR	
Positive	52 (65)
Negative	28 (35)
ISH	
Positive	45 (56.2)
Negative	35 (43.8)
Ki67	
Positive	29 (36.2)
Negative	51 (63.8)
p53	
Positive	12 (15)
Negative	68 (85)
cerbB2	
3+	35 (43.8)
2+	25 (31.2)
0/1+	20 (25)
HER2 expression level (RT-PCR) (mean±SD)	5.7±18.9
p95HER2 expression level (RT-PCR) (mean±SD)	7±2.8

ER: estrogen receptor, PR: progesterone receptor, ISH: in situ hybridization, SD: standard deviation

plification of *HER2 gene* was evaluated with CISH according to nonparametric data. Descriptive analyses of HER2 mRNA expression and p95HER2 expression were evaluated with RT-PCR. In addition, descriptive analyses of patient age, tumor size, number of metastatic lymph nodes, local recurrence, time to metastasis, disease-free survival and overall survival time and follow up time were performed. Vascular and lymphatic invasion, DCIS, perinodal involvement, pT, pN, local recurrence, metastasis, and metastasis to organs were expressed as numeric data. Correlations between variables were tested with x^2 test, Mann-Whitney U test and Spearman's correlation test. ROC estimation was performed. Kaplan-Meier method was performed for disease-free and overall survival and subgroups were compared with log-rank test. A p value <0.05 was accepted as statistically significant.

Results

No patient had signs of distant metastasis at initial diagnosis. Patient characteristics are shown in Table 1. Biomarkers like ER and PgR status, p53 mutant gene, Ki67 proliferation index, HER2 amplification, HER2 and p95HER2 expression levels were analysed in all cases (Table 2). While 43 cases (53.8%) received trastuzumab, 37 (46.2%) did not . Thirty four cases did not receive trastuzumab and did not show amplification with ISH. Forty two cases received trastuzumab and showed amplification with ISH. One case received trastuzumab and did not show amplification with CISH. Three cases did not receive trastuzumab despite its amplification with ISH. HER2 positivity was detected as shown in Figure 1 by CISH.

Mean time to local recurrence/metastasis was 26.23±12.71 months, Mean disease free survival was 44.10±2.2 months and mean follow up time was 48.71±20.1 months. In the last follow up, 71 (88.8%) patients were alive and 9 (11.2%) had died.

Findings belonging to patients who received trastuzumab (with and without trastuzumab resistance)

Trastuzumab was administered to 39 patients. In this group, 28 patients (71.8%) were classified as non-resistant to transtuzumab because no local recurrence or metastasis developed during follow up. Eleven patients (28.2%) developed local recurrence or metastasis and were classified as trastuzumab-resistant.

In these 11 patients with trastuzumab resistance, the mean p95HER2 level was 11.01±19.73.

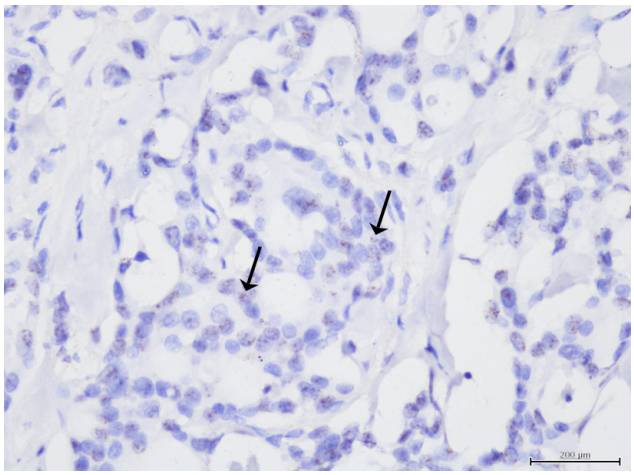

Figure 1. HER2 amplification detected by CISH (x200). Amplified HER2 is shown by multiple brown dots in the nuclei of tumor cells (arrows).

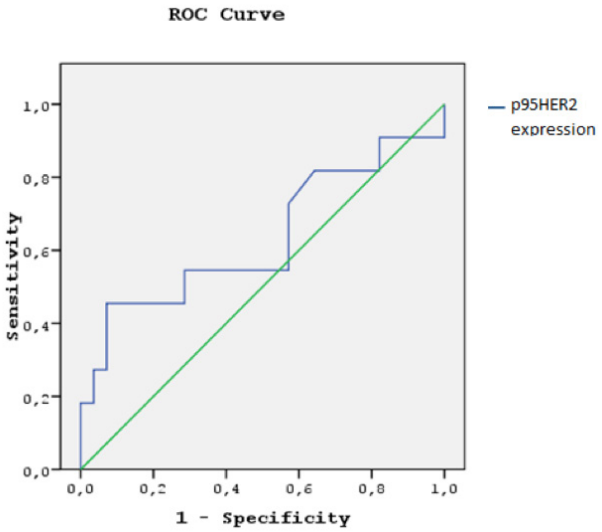

Figure 2. ROC curve of the relation between p95HER2 expression and trastuzumab resistance. The curve is almost linear suggesting good sensitivity.

The mean HER2 level was 0.96±0.72. The mean time to local recurrence/metastasis and mean disease free survival time were the same (20.81±9.00 months). In 28 cases without trastuzumab resistance, the mean p95HER2 level was 1.99±1.37. The mean HER2 expression level was 12.79±30.38, the mean time to the development of local recurrence/metastasis was 39.28±10.90 months and the mean disease free survival was 48.63±20.52 months.

Statistical analysis of cases who received trastuzumab

Statistical analysis of 39 cases with trastuzumab administration was performed. Stage I and II formed one group, stage III and IV formed a second group. According to the defined criteria, in 11 cases with trastuzumab resistance the mean p95HER2 expression level was obviously higher compared to the mean p95HER2 level of 28 cases without trastuzumab resistance, however this difference was not statistically significant (p=0.210, Mann-Whitney U test). Trastuzumab resistance was not detected when the level of p95HER2 expression was low. But whenever p95HER2 level was high, trastuzumab resistance was present. p95HER2 expression levels were sensitive for the detection of trastuzumab resistance, yet not specific (Figure 2).

The mean levels of HER2 expression analyzed by RT-PCR were 0.96±0.72 in trastuzumab resistant cases and 12.79±30.38 in non-resistant cases (p=0.0001, Mann-Whitney U test). The difference of time to local recurrence/metastasis between trastuzumab-resistant and non-resistant cases was highly significant (p=0.005), and the relationship with disease free survival time (p=0.0001) was also statistically significant (Mann-Whitney U test). Amplification curves of p95 and HER2 are shown in Figure 3.

There was a statistically significant relationship (x^2) between trastuzumab resistant and non-resistant cases in terms of lymphatic invasion (p=0.009), nodal involvement (p=0.014), perinodal invasion (p=0.0001), number of metastatic lymph nodes (p=0.013), metastasis (0.0001), lung metastasis (p=0.042) and stage (p=0.011). Results between trastuzumab resistant and non-resistant cases are shown in Table 3.

Discussion

HER2-positive breast cancer is a special molecular and clinical entity which represents a particular group of patients. Treatment of this group of patients with trastuzumab has changed significantly the management and course of the disease. However, it is known that some breast cancer patients who initially responded to trastuzumab show progression later on. Although this highly efficient drug is commonly used, no standard treatment protocol was developed in case of disease progression. Therefore new strategies should be developed as alternative treatments for HER2-positive breast cancer patients who do not respond to trastuzumab.

In HER2-positive breast cancer patients, the role of p95HER2, which is one of the molecular mechanism of resistance to trastuzumab, has been investigated in this study. According to defined criteria, p95HER2 expression didn't show statistically significant difference between trastuzum-

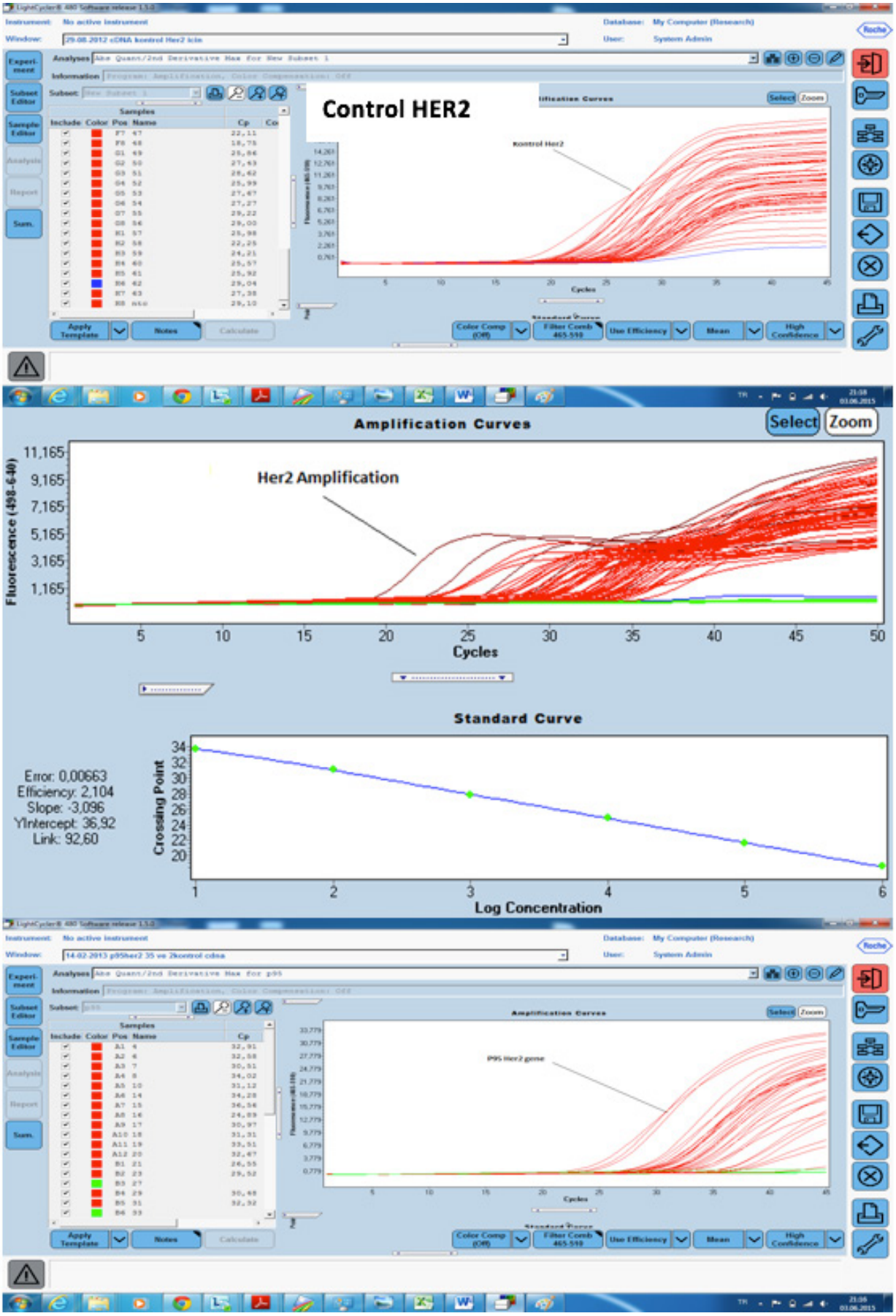

Figure 3. Amplification curves of HER2 and p95HER2 detected by RT-PCR.

Table 3. Comparison of the cases with/without trastuzumab resistance

	Trastuzumab resistance (+) *N (%)*	*Trastuzumab resistance (-)* *N (%)*	*p value*
Age (years), mean±SD	54.64±10.68	51.18±13.59	0.346
Tumor size (cm), mean±SD	3.17±1.28	2.42±1.49	0.034
HER2 expression, mean±SD	0.9±0.7	12.8±30.3	0.0001
p95HER2 expression, mean±SD	11.0±1.0	1.0±1.3	0.210
DFS (months), mean±SD	20.8±9.00	48.6±20.52	0.0001
ER pos	6(54.5)	20(71.4)	0.453
PgR pos	5(45,5)	19(67.9)	0.277
Ki67 pos	9(81.8)	11(61.1)	0.412
Vascular invasion	4(36.4)	7(25)	0.694
Lymphatic invasion	11(100)	16(57.1)	0.009
Nodal involvement	10(90.9)	13(46.4)	0.014
Perinodal invasion	11 (100)	10(35.7)	0.0001
Stage I-II	2(18.2)	19(67.8)	0.011
Stage III-IV	9(81.8)	9(32.1)	0.011
Local recurrence	11(100)	2(7.1)	0.001
Metastasis	11(100)	6(21.4)	0.0001

DFS: disease free survival, SD: standard deviation

ab-resistant and non-resistant groups. However, trastuzumab resistance was not detected in groups expressing low levels of p95HER2. Besides, whenever p95HER2 was highly expressed, trastuzumab resistance was detected. This result showed that p95HER2 expression levels were sensitive but not specific in determining of trastuzumab resistance.

HER2 expression levels were significantly higher in the trastuzumab-resistant group compared to the non-resistant group (p=0.0001). This observation led us to suppose that p95HER2 overexpression could possibly suppress the p185HER2 (full length HER2) of HER2 expression in trastuzumab-resistant patients. The ratio between these two levels (of p95HER2 and p185HER2) or proportional change may carry more statistical significance for determination of resistance. The data over this subject will become clearer as more studies which will contain estimations of these two levels in trastuzumab-resistant and non-resistant cases will be conducted.

In the literature, no study could be found comparing the relationship between trastuzumab-resistant and non-resistant subgroups on the basis of quantitative values of both HER2 and p95HER2 expression levels in HER2-positive primary breast carcinoma. A certain threshold value is determined as positive in conducted studies [14,15]. Sperinde et al. made an immunohistochemical evaluation in paraffinized breast cancer tissues, thanks to an antibody that they developed against p95HER2 protein in laboratory conditions [14]. In this study they determined the level of p95HER2 expression by VeraTag technology as well and found that high p95HER2 expression levels were significantly correlated with reduced disease free survival (p=0.022) and overall survival (p=0.009). The authors emphasized that IHC was not as sensitive and quantitative as VeraTag technology in terms of p95HER2 assessment method [14]. Kocar et al. [15] also found that the IHC methods may have been inadequate for determining p95HER2 expression levels.

Full length HER2 expression levels are similar in primary and metastatic breast cancer. However, p95HER2 expression level shows significant increase in metastatic lymph nodes compared to the expression in the primary tumor and this situation demonstrates the role of p95HER2 in disease progression and metastasis [8]. In a study investigating the prognostic value of p95HER, Saez et al. evaluated the levels of HER2 and p95HER2 by Western blot analysis. In this study, the authors determined HER2 and p95HER2 levels as ''high level'' or ''low level'' and found that high level of p95HER2 expression was highly correlated with decreased disease free survival (p=0.0001) but high HER2 level was not correlated with disease free survival (p=0.261) in the primary tumor [8]. In our study, p95HER2 levels were not divided

into two groups as ''high'' and ''low'' based on a treshold level. Assessment of data about disease free survival and overall survival parameters was a parametric value. HER2 and p95HER2 expression levels were parametric measurement data as well. For all patients involved in study, both HER2 and p95HER2 expression levels were not correlated with decreased disease free survival (p=0.071 and p=0.517, respectively). However, in our study, a statistically significant relationship (p=0.0001) between high p95HER2 expression levels and decreased disease free survival was detected in the trastuzumab-resistant group.

p95HER2 overexpression co-occurs with reduced trastuzumab response in xenografts and human breast cancers [9]. In this retrospective analysis, significantly reduced response rates to trastuzumab and trastuzumab- chemotherapy combination were detected in p95HER2-expressing patients compared to patients expressing p185HER2 (full length HER2). p95HER2 was detected by immnunofluorescent method and Western blot analysis and consistent results were obtained. However, as it is known, these evaluation methods calculate the level of proteins. Instead of these methods, more feasible methods with high repeatability are needed in daily routine practice. Regarding all patients enrolled in this study, a nonsignificant relationship between p95HER2 expression level and lymphatic invasion (p=0.191), perinodal involvement (p=0.646), number of metastatic lymph nodes (p=0.744), metastasis (p=0.179) and disease free survival (p=0.517) was present. These results raised a question that another parameter may affect the statistical outcome in the group containing all of the patients.

Duchnowska et al. quantitatively evaluated the expression of p95 protein both in the primary tumor and brain metastasis of patients who developed such metastasis by using VeraTag [13]. In this study, the mean p95HER2 expression was 1.5-fold higher than in the primary tumor (p=0.007) [13]. In our study, there were 2 patients with brain metastasis. The level of p95HER2 expression in their primary tumor was 1.43 (case no. 76) and 0.59 (case no. 77). When the mean p95HER2 expression levels were compared with trastuzumab-resistant and non-resistant patients, nearly 5-fold increase was detected in trastuzumab-resistant patients. However, our evaluation covered only the primary tumor. Expression analysis of p95HER2 was not performed in metastatic tumors. Duchnowska et al. assessed the total HER2 protein expression as well as p95HER2 expression [13] and they made this assessment by using HERmark measurements defined by Huang et al. [16]. In the primary breast cancer and in brain metastasis of the same tumor, Duchnowska et al. found that the quantitative levels of HER2 expression were 2.1-fold higher (p<0.0001) in brain metastasis to the matched primary breast cancer [13]. As a result of this study, broad-based clinical studies with breast cancer patients require quantitative measurements of HER2 and p95HER2 along with other biomarkers.

Conclusion

Significantly reduced response rates to trastuzumab and trastuzumab ± chemotherapy combination were detected in p95HER2-expressing patients compared to patients expressing p185HER2 (full length HER2). Also, according to the literature, it is understood that overexpression of p95HER2 possibly influences the progression of HER2-positive tumors and therefore, use of trastuzumab does not meet a targeted treatment requirements in such patients. All these said, we finally claim that, in breast cancer, evaluation of either p95HER2 or p95HER2 with HER2 may be beneficial before the initiation of trastuzumab when planning the treatment of HER2-positive breast cancer patients.

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