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ORIGINAL ARTICLE

p95-HER2 and trastuzumab resistance in metastatic breast cancer; is immunohistochemistry appropriate?

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Summary

Purpose: *Unraveling the mechanisms underlying the resistance to trastuzumab is important for amending the prognosis of patients with human epidermal growth factor receptor 2 (HER2) positive metastatic breast cancer. Experimentally, it has been shown that p95-HER2 positive breast tumors are resistant to trastuzumab. The aim of this study was to investigate the predictive and prognostic importance of p95-HER2 expression by immunohistochemistry in HER2-positive metastatic breast cancer patients treated with trastuzumab.*

Methods: *Only patients who had a histological diagnosis of HER2-positive metastatic breast cancer and who had received first line therapy containing trastuzumab were enrolled in the study. Immunohistochemistry was used to analyze p95-HER2 expression in the tissue blocks of the patients.*

Results: *The study was performed on 38 patients aged between 30 and 84 years. In 14 patients (36.8%), p95-HER2 was positive, whereas it was negative in the remaining 24 patients (63.2%). There was no significant correlation between p95-HER2 expression and overall survival, response to trastuzumab, and progression-free survival (PFS).*

Conclusion: *Unlike previous reports, there was no correlation between the p95-HER2 expression and resistance to trastuzumab. It may be argued that an analysis using immunohistochemistry is inadequate for determining p95-HER2. In order to ascertain whether immunohistochemistry is an appropriate method, studies with larger patient groups are needed.*

Key words: *breast cancer, HER2, p95-HER2, trastuzumab*

Introduction

Breast cancer is the most common cancer and the leading cause of cancer-related deaths in females. Identifying the genetic background of breast cancer and having a better understanding of the underlying molecular mechanisms have enabled an increase in disease-related diagnostic methods, prophylaxis strategies, and treatment opportunities. The discovery of the estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2), which are specific therapeutic targets for breast cancer, has been the most crucial development in the therapy of breast cancer patients with these receptors.

Approximately 20% of the newly diagnosed breast cancer patients have HER2 amplification and/or overexpression, and the presence of HER2 has been found to be correlated with a more aggressive clinical course and decreased survival rates when compared with patients with normal HER2 levels [1]. Trastuzumab, which binds to the extracellular domain of HER2, is a human monoclonal antibody. When used as a single agent, it provides a clinical response, and when combined with chemotherapy, it increases survival rates and diminishes the recurrence rate by approximately 50% in the adjuvant therapy of HER2-overexpressed tumors [2]. However, not all HER2-positive breast cancer patients respond to

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trastuzumab treatment. Although p95HER2, the enzymatically-cleaved form of the HER2 receptor, has kinase activity, it lacks an extracellular domain and trastuzumab binding site. Node-positive patients express higher levels of p95-HER2 than node-negative patients [3]. Although in vitro data claims that trastuzumab prevents the breakdown of p185HER2 (full length HER2) and, as a result, the production of p95-HER2, a retrospective study revealed a relationship between the existence of p95-HER2 and clinical resistance to trastuzumab therapy [4]. In pharmacokinetic studies and preclinical models the clearance of the extracellular domain in complex with trastuzumab was shown to be faster than for the clearance of trastuzumab alone [5,6]. For a long time the HER2 extracellular domain has been regarded as a potential predictive factor for response to therapy. However, in a meta-analysis in which patients with metastatic breast cancer on trastuzumab therapy from 4 clinical studies were collected, it was revealed that both the extracellular domain baseline level and the extracellular domain decrease following therapy have a low predictive value for the clinical benefits of trastuzumab therapy [7].

Exploring the mechanisms that underlie trastuzumab resistance is crucial for amending the prognosis of HER2-positive metastatic breast cancer patients. Our purpose was to assess the predictive and prognostic importance of p95-HER2 expression by immunohistochemistry in HER2-positive metastatic breast cancer patients treated with trastuzumab.

Methods

Patients with a histological diagnosis and HER2-positive metastatic breast cancer and who were receiving treatments that included trastuzumab (8 mg/kg i.v. day 1, followed by 6 mg/kg i.v. every 21 days) were enrolled in this retrospective study, which was approved by the local ethics committee. Patients with positive hormone receptor (ER or progesterone receptor/PR), soft tissue or bone metastases received antihormonal therapy (tamoxifen 20 mg p.o. daily for premenopausal patients, and letrozole 2.5 mg p.o. daily, anastrozole 1 mg/p.o. daily or exemestane 25 mg p.o. daily for postmenopausal patients). Hormone receptor negative patients with soft tissue or bone metastases received capecitabine 1250 mg/m^2 twice daily, days 1-14, every 21 days. All patients with visceral metastases, regardless of hormone receptor status, received taxane-containing regimens. Immunohistochemistry was used to screen for p95-HER2 expression in paraffin breast cancer tissue blocks of the patients. In addition, all dyed sections were blindly reviewed by a pathologist without knowing the histological grade, hormone receptor status, or HER2 results. The differences between histological grade, hormone receptor status, objective response to trastuzumab, PFS, and overall survival (OS) between the group with p95-HER2 expression and the group without this expression were investigated. OS was determined as the time from the start of treatment including trastuzumab until death or the last follow-up. PFS was regarded as the time from the date when the treatment including trastuzumab started until the date when disease progression was detected. Response to trastuzumab-based therapy was evaluated every 2-3 months by using the modified Response Evaluation Criteria in Solid Tumors (RECIST) guidelines.

Immunohistochemical method

In formalin-fixed tissues, the streptavidin-biotin-peroxidase immunohistochemical dye method was used in order to detect p95-HER2 immunoexpression. In this method, 3μm-thick sections were transferred to positively charged microscope slides from paraffin-embedded tissues and were deparaffinized overnight at 37°C. Then, the deparaffinization was completed by soaking them for 5 min in 3 separate xylenes. The sections were soaked in 2 separate 96% ethanol solutions, and the endogenous tissue peroxidase activity was repressed by a 3% hydrogen peroxide solution. In order to reveal the masked antigens, an antigen recovery procedure was used by placing a pH 6 citrate buffer solution in a microwave oven and applying it to the sections washed by distilled water before. The microscope slides that had been cooled at room temperature for 20 min were washed with 2 separate phosphate buffer solutions (PBS) and a 10-min protein blockage (Histostain Bulk Kit, Invitrogen Ltd., Paisley, UK) was performed on the tissues in order to prevent non-specific dying. Following the blockage, 1:1000 diluted anti-p95 NBS1 (phospho S432) antibody (EPR2470Y, Abcam, Cambridge, USA) was dripped on separate cross sections and incubated for 30 min at room temperature. At the end of this time, the cross sections were washed with 2 different PBS and were kept in a biotinized secondary antibody (Histostain Bulk Kit, Invitrogen Ltd., Paisley, UK) for 10 min. Following another wash with the PBS, streptavidin-peroxidase (Histostain Bulk Kit, Invitrogen Ltd., Paisley, UK) was dripped onto the cross sections, and a 10-min incubation followed. The cross sections that were washed with PBS and dye had 3.3'-diaminobenzydine (DAB) chromogen dripped on them, and this was checked after 5-min incubation. Converse dyeing was performed with Mayer's hematoxylin to the cross sections that had been washed with distilled water. They were then dehydrated via ethanol. The tissues that had been covered with appropriate covering material were put into xylene and evaluated by light microscope (BX51, Olympus Corporation, Tokyo, Japan). If the tumor tissue had more than 10% membranous staining, p95-HER2 was considered to be positive [8]. Examples of p95-HER2 staining are shown in Figure 1.

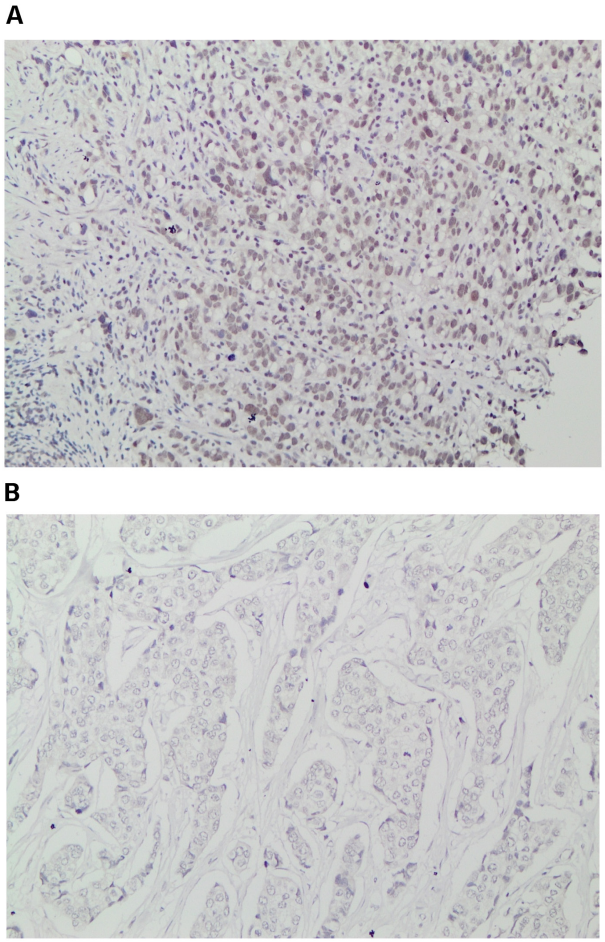

Figure 1. A: an example of intensive p95-HER2 staining (p95-HER2 immunohistochemical stain x20). **B:** an example of negative p95-HER2 staining (p95-HER2 immunohistochemical stain x20).

Statistics

The Number Cruncher Statistical System (NCSS) 2007 and Power Analysis and Sample Size (PASS) 2008 statistical software (NCSS LLC, Kaysville, USA) programs were used for statistical analysis. Mann-Whitney U test was used for comparing the quantitative data as well as definitive statistical methods (mean, standard deviation, median, frequency, and ratio). The chi-square test, Yates' chi-square test, and Fisher's exact test were used for comparing the qualitative data. The Kaplan-Meier method and log-rank test were used for survival analyses. A value of $p<0.05$ was considered to be statistically significant.

Results

The study included a total of 38 patients who had HER2-positive metastatic breast cancer. The patient age ranged between 30-84 years (median 49). The patient and disease characteristics and the therapies administered are shown in Table 1.

When the responses to trastuzumab were reviewed, it was seen that 23.7% (N=9) of the patients had progressive disease, 50% (N=19) had partial response, 18.4% (N=7) had complete response, and the remaining 7.9% (N=3) had stable disease as best response No significant differences were seen between positive and negative p95-HER2 patients in terms of response (Table 2).

In p95-HER2 positive patients the median PFS was 11.16 months (range 2.91-35.5). The corresponding figures in p95-HER2 negative patients were 12.37 months (range 2.95-29.99). In p95-HER2 positive patients the median OS was 27.66 months (range 10.01-51.5). The corresponding figures in p95-HER2 negative patients were 24.02 months (range 10.38-41.98).

Again, no significant differences were noted between positive and negative p95-HER2 patients in terms of survival (Table 3).

Table 1. Patient and disease characteristics and therapies administered

Characteristics and therapies	*N (%)*
Menopausal status	
Postmenopausal	24 (63.2)
Premenopausal	14 (36.8)
Histological grade	
1	1 (2.6)
2	23 (60.5)
3	14 (36.8)
Hormone receptors (ER or PR)	
Positive	14 (36.8)
Negative	24 (63.2)
Metastatic location	
Bone/soft tissue	19 (50)
Visceral	19 (50)
Additional therapy with trastuzumab	
Anti-hormonal	6 (15.8)
Capecitabine	3 (7.9)
Taxane-based	29 (76.3)

Table 2. Trastuzumab response according to p95-HER2

Trastuzumab response	*p95-HER2 positive N (%)*	*p95-HER2 negative N (%)*	*p-value**
Progression	5 (35.7)	4 (16.7)	0.245
Partial response	8 (57.1)	11 (45.8)	0.737
Complete response	1 (7.1)	6 (25.0)	0.227
Stable disease	0	3 (12.5)	0.283

* Fisher's exact test

Table 3. Survival according to p95-HER2

	p95-HER2 positive	*p95-HER2 negative*	*p-value**
Median PFS (mo)	11.16	12.37	0.525
Median OS (mo)	27.66	24.02	0.555

OS: overall survival, PFS: progression free survival, mo: months
*Mann-Whitney U test

p95-HER2 was positive in 36.8% (N=14) of the patients and negative in the remaining 63.2%. There were no statistically significant differences between p95-HER2 and grade. In addition, there was no statistically relevant correlation between hormone receptor status rates and p95-HER2 levels, and no statistically significant differences existed between p95-HER2 and the metastatic locations (Table 1).

When the overall survival rates were assessed by the log-rank test according to p95-HER2 status, no statistically significant differences were found in 5-year survival rates (p>0.05; Figure 2).

Discussion

The proteolytic breakdown of the HER2 extracellular domain has been studied as a resistance mechanism to monoclonal antibody-based therapies, and it was discovered that even though p95-HER2 lacks an extracellular domain and trastuzumab binding site, it does possess kinase activity. According to the literature, approximately 25% of the HER2-overexpressed breast cancer cases expresses this form of the receptor [9]. When p95-HER2 positivity in our study was compared with other relative studies [4,8,9], it was observed to be relatively high. This may be due to the fact that the present study was performed using immunohistochemistry or because the size of the study group was small.

Although it has been claimed in *in vitro* models that trastuzumab prevents p185-HER2 breakage, which causes a halt in p95-HER2 production, a retrospective study reported association between the presence of p95-HER2 and the clinical resistance to trastuzumab therapy [10]. The p95-HER2 levels of 46 metastatic breast cancer patients were measured by the immunofluorescence method in that study, and only 1 patient out of the 9 (11.1%) with positive p95-HER2 expression responded to trastuzumab therapy. On the other hand, 19 of the 37 patients (51.4%) who had positive p185-HER2 expression responded to the same therapy [4] .

The proteolytic cleavage of full-length HER2 by metalloproteinases generates a 95 to 100 kDa p95-HER2 fragment that could be cleaved by gamma-secretase to generate a 90 to 95 kDa p95-HER2 fragment. The 100 to 115 kDa p95-HER2 fragment is constitutively hyperactive because of its ability to form dimers maintained by intermolecular disulfide bonds. The activity of the 95 to 100 kDa fragment is comparable to that of full-length HER2, whereas the soluble intracellular fragments are inactive [11-13]. Previous studies on p95-HER2 have analyzed the presence of fragments ranging from 90 to 115 kDa via Western blot analysis [14,15]. Samples were scored positive if any fragments within that range were detected at levels above a certain cutoff. Therefore, in principle, those studies did not distinguish between samples expressing only inactive p95-HER2 fragments of 90 to 95 kDa and samples expressing only active p95-HER2 fragments of 95 to 100 or 100 to 115 kDa, or any intermediate combination of these possibilities. A study by Sperinde et al. in which p95-HER2 was directly determined by quantitative methods showed a positive correlation between high p95-HER2 levels and shorter PFS and OS rates [8].

Saez et al. investigated the prognostic importance of p95-HER2 and reported that p95-HER2 levels associated with decreased survival [9]. The 5-year disease-free survival rates were lower in patients with higher levels of p95-HER2 expression.

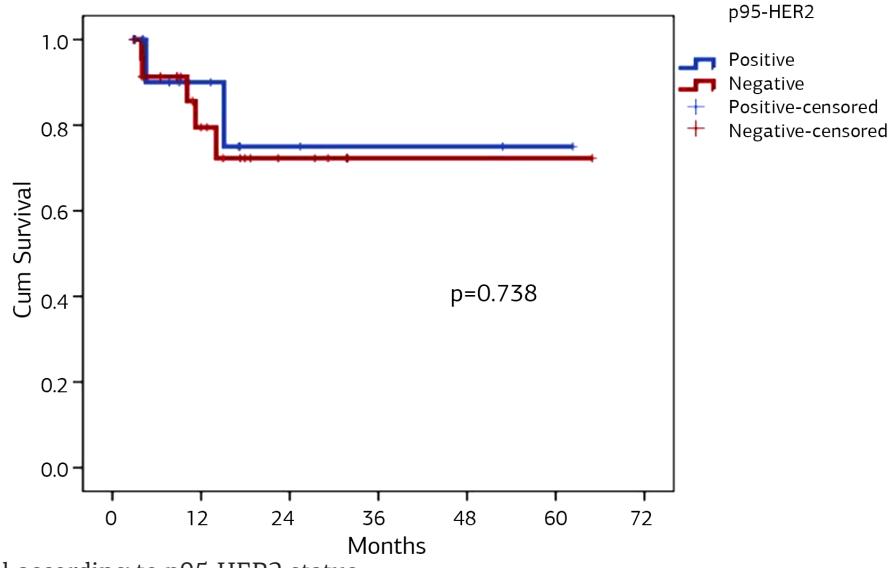

Figure 2. Overall survival according to p95-HER2 status.

In this study in which 483 patients with primary breast cancer were evaluated, it was determined that the median disease-free survival period was 32 months in patients with high levels of p95-HER2 expression while it was 139 months in patients with low levels of p95-HER2 expression.

Molina et al. conducted a study in which the p95-HER2 and p185-HER2 levels of 337 breast cancer tissue and 81 metastatic lymph nodes were evaluated using the Western blot technique, and it was pointed out that node-positive patients had higher rates of p95-HER2 expression than node-negative ones [3]. In this technique, which has only recently been used to identify the presence of p95-HER2 in breast cancer, large quantities of fresh tumor tissue are needed. However, this method has serious limitations due to the fact that appropriate fresh tissue samples can rarely be obtained from clinical samples. In order to overcome this problem, a study using the immunofluorescence method in formalin-fixed paraffin-embedded tissues was designed. The positive predictive value of this method was 100%, and the negative predictive value 94% [4].

In our study, p95-HER2 was positive in 36.8% (N=14) of the patients and negative in 63.2% (N=24). The detection of non-active subtypes may explain the high rate of p95-HER2 positivity. There were no statistically significant differences between p95 and grade, hormone receptor status, or metastatic regions. Moreover, we found no statistically significant correlations between p95-HER2 levels and OS, trastuzumab responses, or PFS. This may be attributed to our limited number of patients, or it could be that the immunohistochemical methods may have been inadequate for determining p95-HER2 levels. Immunohistochemistry is faster, cheaper and more easily accessible than other methods. However, false positive and negative results are frequently encountered. In order to ascertain whether immunohistochemistry is an appropriate method for evaluating p95-HER2, future studies with more subjects should be conducted.

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