Quantitative Measurements of Tumoral p95HER2 Protein Expression in Metastatic Breast Cancer Patients Treated with Trastuzumab: Independent Validation of the p95HER2 Clinical Cutoff

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Abstract

**Purpose:** P95HER2 (p95) is a truncated form of the HER2, which lacks the trastuzumab-binding site and contains a hyperactive kinase domain. Previously, an optimal clinical cutoff of p95 expression for progression-free survival (PFS) and overall survival (OS) was defined using a quantitative VeraTag assay (Monogram Biosciences) in a training set of trastuzumab-treated metastatic breast cancer (MBC) patients.

**Experimental Design:** In the current study, the predictive value of the p95 VeraTag assay cutoff established in the training set was retrospectively validated for PFS and OS in an independent series of 240 trastuzumab-treated MBC patients from multiple institutions.

**Results:** In the subset of 190 tumors assessed as HER2-total (H2T)-positive using the quantitative HERmark assay (Monogram Biosciences), p95 VeraTag values above the predefined cutoff correlated with shorter PFS (HR = 1.43; P = 0.039) and shorter OS (HR = 1.94; P = 0.0055) where both outcomes were stratified by hormone receptor status and tumor grade. High p95 expression correlated with shorter PFS (HR = 2.41; P = 0.0003) and OS (HR = 2.57; P = 0.0025) in the hormone receptor-positive subgroup of patients (N = 78), but not in the hormone receptor-negative group. In contrast with the quantitative p95 VeraTag measurements, p95 immunohistochemical expression using the same antibody was not significantly correlated with outcomes.

**Conclusions:** The consistency in the p95 VeraTag cutoff across different cohorts of patients with MBC treated with trastuzumab justifies additional studies using blinded analyses in larger series of patients. *Clin Cancer Res; 20(10); 1–9. ©2014 AACR.*

Introduction

P95 was first identified as a band on a HER2 Western blot analysis, migrating at approximately 95 kilodaltons (1), and was subsequently shown to be a poor prognostic factor in HER2-positive breast cancer patients before the era of trastuzumab (2). P95 was later shown to be a resistance marker for trastuzumab (3), a monoclonal antibody used to treat HER2-positive breast cancer. This is consistent with the fact that p95 lacks the trastuzumab-binding site, but retains kinase activity.

More recently, a number of different HER2 carboxy terminal fragments (CTF) have been identified in breast cancers, but each of these HER2-CTFs showed strikingly different activities. The most abundant HER2-CTF, identified as M687-HER2-CTF, seems to be inactive (4). In contrast, the M611-HER2-CTF form is present in lesser abundance but is hyperactive, possibly due to its tendency to covalently homodimerize through unpaired cysteines (4). This form is likely responsible for the detrimental effects associated with what has become known as "p95," including potential resistance to trastuzumab (5).

At least two antibodies have been raised against the M611-HER2-CTF form of p95 (6, 7), as reviewed by Arribas and colleagues (8, 9). One of these antibodies, D9, has been

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-13-2782

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Translational Relevance

P95 is a hyperactive, truncated form of HER2 that lacks the binding site for trastuzumab and pertuzumab. It is variably expressed in HER2-positive breast cancers and is thought to be a marker for trastuzumab-resistance when expressed at high levels. A quantitative p95 assay, specific for the active M611-HER2 carboxy-terminal fragment, was previously used to identify a p95 cutoff for patients with metastatic breast cancer (MBC) with poor progression-free survival and overall survival while receiving trastuzumab-containing therapy. In the current study, this prespecified p95 cutoff was validated in an independent set of 240 patients with MBC collected from multiple institutions. The predictive value of p95 was particularly strong in the hormone receptor-positive patients. Because p95 does not seem to predict resistance to HER2 tyrosine kinase inhibitors (TKI), a quantitative p95 assay may prove useful in identifying patients who would most benefit from the addition of a HER2 TKI to therapy containing a HER2-directed antibody or other anti-HER2 strategies.

Materials and Methods

Patient samples

Formalin-fixed, paraffin-embedded (FFPE) primary tumors from 240 trastuzumab-treated MBC patients were obtained from the authors’ institutions. Patient characteristics are shown in Table 1. Trastuzumab was administered on the basis of the original determination of HER2 positivity as HER2 IHC 3+ or a HER2/CEP17 FISH ratio ≥ 2. Trastuzumab-containing therapy was generally continued until progression, with a median duration on trastuzumab of 10 months. Most patients (84%) received chemotherapy throughout the entire study period. Estrogen receptor alpha (ER-α) and progesterone receptor (PR) expressions were determined by immunohistochemistry (IHC), with ≥10% nuclear staining considered positive. Hormone receptor positivity was defined as either ER-α or PR positivity. This study was approved by the Ethics Committee of the Medical University in Gdańsk (Gdańsk, Poland), the coordinating center. All patient data were anonymized before analysis.

Quantitative HER2 assay

Investigators who performed the HERmark and p95 assays were blinded to all clinical and histopathologic data used to build a quantitative p95 assay using the VeraTag platform (6).

In a previous study, an optimal p95 VeraTag cutoff for progression-free survival (PFS) and overall survival (OS) was established in a cohort of trastuzumab-treated metastatic breast cancer (MBC) patients (6). The current independent cohort was assembled to validate this prespecified p95 cutoff.

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All patients</th>
<th>HR negative</th>
<th>HR positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age at the start of trastuzumab-containing therapy</td>
<td>54 (55)</td>
<td>55 (91)</td>
<td>53 (90)</td>
</tr>
<tr>
<td>Stage at presentation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I–III</td>
<td>217 (90)</td>
<td>123 (91)</td>
<td>94 (90)</td>
</tr>
<tr>
<td>Stage IV</td>
<td>23 (10)</td>
<td>12 (9)</td>
<td>11 (10)</td>
</tr>
<tr>
<td>HER2 level by HERmark</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (H2T ≤ 13.8)</td>
<td>50 (21)</td>
<td>23 (17)</td>
<td>27 (26)</td>
</tr>
<tr>
<td>Overexpression (H2T &gt; 13.8)</td>
<td>190 (79)</td>
<td>112 (83)</td>
<td>78 (74)</td>
</tr>
<tr>
<td>FISH/CEP17 (4 unknown)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤2.0</td>
<td>37 (16)</td>
<td>13 (10)</td>
<td>24 (23)</td>
</tr>
<tr>
<td>&gt;2.0</td>
<td>199 (84)</td>
<td>118 (90)</td>
<td>81 (77)</td>
</tr>
<tr>
<td>Tumor grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1 (well differentiated)</td>
<td>4 (2)</td>
<td>1 (1)</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Grade 2 (moderately differentiated)</td>
<td>97 (40)</td>
<td>45 (33)</td>
<td>52 (50)</td>
</tr>
<tr>
<td>Grade 3 (poorly differentiated)</td>
<td>139 (58)</td>
<td>89 (66)</td>
<td>50 (48)</td>
</tr>
<tr>
<td>Hormone receptor status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER-α and PR negative</td>
<td>135 (56)</td>
<td>135 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>ER-α or PR positive</td>
<td>105 (44)</td>
<td>0 (0)</td>
<td>105 (100)</td>
</tr>
<tr>
<td>Pathology type (8 unknown)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ductal</td>
<td>212 (91)</td>
<td>119 (91)</td>
<td>93 (91)</td>
</tr>
<tr>
<td>Lobular</td>
<td>13 (6)</td>
<td>5 (4)</td>
<td>8 (8)</td>
</tr>
<tr>
<td>Other</td>
<td>7 (3)</td>
<td>6 (5)</td>
<td>1 (1)</td>
</tr>
</tbody>
</table>

NOTE: HR positivity defined as either ER-α or PR positivity (≥10% positive nuclear staining).
until H2T and p95 results were reported to the Coordinating Center. Total HER2 protein expression (H2T) was measured using the HERmark assay (Monogram Biosciences), as previously described (10, 11). The HERmark assay provides a tumor averaged HER2 measurement much the same way HER2 ISH assays are designed to provide a tumor averaged gene copy number. Briefly, H2T was quantified through the proximity-based release of a fluorescent tag (\textit{vTag} for “VeraTag reporter”, see Fig. 1A) conjugated via a thioether linker to a HER2 monoclonal antibody (mAb), Ab-8 (Thermo Fisher). The antibody was paired with a biotinylated second HER2 mAb, Ab-15 (Thermo Fisher), linked to a streptavidin methylene blue photosensitizer molecule (PM). Upon illumination with red light, the PM produced singlet oxygen (\(1O_2\)), which cleaved only the thioether linkers in close proximity, liberating VeraTag reporter molecules. Signal (\(vTag\)), quantified by capillary electrophoresis was normalized to invasive tumor area on the FFPE tissue section. Multiple cell line controls were included in each batch for normalization. Analytical cutoffs aligned to central HER2 determination had been determined previously to define HERmark-negative values (H2T < 10.5 relative fluorescence/mm² tumor; RF/mm²) and HERmark-positive values (H2T > 17.8 RF/mm²) with equivocal values defined as 10.5 \(\leq\) H2T \(\leq\) 17.8. These cutoffs were derived from the <5th percentile of centrally determined HER2 positives and the >95th percentile of centrally determined HER2 negatives, respectively, within a reference database of 1,090 breast cancer patient samples. The cutoff of 13.8 RF/mm² that best discriminated better versus worse patient outcomes on trastuzumab-based therapy was earlier determined in an independent cohort of patients (12). This cutoff was used in all clinical analyses in the current study to define HER2 positivity by the VeraTag assay.

**Quantitative p95 assay**

The VeraTag p95 assay method and proprietary p95 monoclonal antibody (D9, Monogram Biosciences) characterization was previously described (6). D9 specifically recognizes the highly active M611-HER2-CTF form of truncated HER2, but is likely sterically blocked from binding to full-length HER2 (Fig. 1B). D9 binding to the FFPE tumor section was detected by a secondary antibody conjugated to a fluorescent VeraTag reporter molecule. The VeraTag reporter was released by reduction and quantified by capillary electrophoresis. Multiple cell line controls were included in each batch for normalization. Similar to the HERmark assay, relative fluorescence was normalized to tumor area to give units of RF/mm². The prespecified p95 \(\geq 2.8\) RF/mm² cutoff, derived from an independent training set, as previously described (6), was used to define p95 positivity in the current study.

**P95 IHC**

p95 expression was assessed by IHC using the same D9 antibody as the p95 VeraTag assay. The p95 immunohistochemical assay was run on a Ventana Discovery XT autostainer using the extended retrieval setting, 2.5 mg/mL D9 anti-p95 and OmniMAP DAB Kit. Four cell line controls were included in each batch that spanned the range of 0 (negative, no staining) to 3 (intense staining). p95 IHC was scored categorically, according to a staining intensity scale from 0 to 3, with at least 10% of the cells staining in the highest category. P95 IHC H-score was computed as the sum of \(3 \times [\% 3+] + 2 \times [\% 2+] + 1 \times [\% 1+]\).

**Statistical analysis**

The principal aim of the current study was validation of the prespecified p95 cutoff of 2.8 RF/mm² for PFS and OS in an independent cohort of HER2-positive MBC patients treated with trastuzumab. The Kaplan–Meier method and the log-rank test for \(P\) values with stratification by hormone receptor status and tumor grade (1/2 vs. 3) were used for analysis of PFS and OS. In analyses on hormone receptor-positive or -negative subsets, only tumor grade was used as a stratification factor. PFS and OS were measured from the time of initiation of trastuzumab-containing therapy. P95...
expression was also assessed by IHC using the same antibody as the p95 VeraTag assay, and correlations with PFS and OS were calculated using the same methods. The Mann–Whitney test was used to calculate $P$ values for differences in distributions of p95 or H2T in grade or hormone receptor status subgroups.

Results

Relationships between p95 protein, HER2 protein, and HER2 FISH

HER2 status was originally determined at the local institution by IHC and, for samples showing intermediate expression (scored 2 +), by FISH. Subsequently, HER2 FISH status was centrally determined for all but 10 patients. The FISH HER2/CEP17 ratio and quantitative HER2 expression (H2T) measurements followed a log-log relationship (Fig. 2A) with a Pearson $R^2$ of 0.45 ($P < 10^{-13}$), after excluding cases with FISH signals too dense to count, designated as $<LOD$. The p95 clinical cutoff is shown at 2.8 RF/mm². C, p95 versus H2T with clinical cutoffs described in A and B. Relationship between H2T and clinical variables. D, median H2T is greater in the hormone receptor-negative subset ($P = 0.0047$). E, median H2T is greater in the grade 3 subset ($P = 0.020$). F, median p95 is greater in the hormone receptor-negative subset ($P < 0.001$). G, median p95 is greater in the grade 3 subset ($P < 0.001$).

The relationship between VeraTag p95 protein expression and FISH HER2/CEP17 is shown in Fig. 2B. Samples with p95 below the limit of detection (LOD) were placed at the bottom of the graph and labeled as $<LOD$. Tumors with nonamplified HER2 (HER2/CEP17 $< 2$) expressed low levels of p95, generally below the prespecified p95 cutoff of 2.8 RF/mm². In contrast, 57% of tumors with amplified HER2 were p95 high (p95 $> 2.8$ RF/mm²), with little dependence of p95 level on the HER2/CEP17 ratio (Pearson $R^2 = 0.029$; $P = 0.023$).

Overall, the correlation between p95 and H2T (Fig. 2C) was low (Pearson $R^2 = 0.20$; $P < 10^{-10}$). Expression of p95 was limited at low H2T values, but spanned an approximately 20-fold range at high H2T values. In tumors with HER2 protein overexpression (H2T $\geq 13.8$ RF/mm²), 60% had high p95.

Correlation of p95 and H2T with hormone receptor status and grade

Both H2T and p95 were negatively correlated with hormone receptor status and positively correlated with tumor...
grade (Fig. 2D–G). Although the correlations were all statistically significant, the differences in the medians were small, in the range of 1.4-fold to 1.7-fold.

Correlation of p95 with progression-free survival and overall survival

The primary aim of the current study was validation of the p95 cutoff at 2.8 RF/mm², established in a training set of trastuzumab-treated MBC patients (6). As in the training set, the p95 analysis was restricted to patients with HER2-positive tumors, as determined by the VeraTag assay (H2T > 13.8 RF/mm²). Most of the cases that were HER2 negative by VeraTag were also HER2 negative by FISH (Fig. 2A).

A p95 cutoff of 2.8 RF/mm² was established in the training set as best discriminating the groups of patients with different PFS and OS. In the current set, the predictive value of the same cutoff was confirmed for both PFS (HR = 1.43; P = 0.039) and OS (HR = 1.94; P = 0.0055; Table 2 and Fig. 3A and B).

Because of the relationship of p95 expression and ER expression (7), we assessed p95 correlations with PFS and OS separately for hormone receptor-negative and -positive subgroups. Among patients with hormone receptor-negative tumors, p95 was not predictive for either PFS (HR = 0.95; P = 0.82) or OS (HR = 1.38; P = 0.34; Table 2; Fig. 3C and D). Conversely, among patients with hormone receptor-positive tumors, p95 showed strong predictive value for both PFS (HR = 2.41; P = 0.0003) and OS (HR = 2.57; P = 0.0025; Table 2).

P95 IHC

In the current study, 229 of the 240 cases had sufficient tumor tissue for the p95 immunohistochemical assay. Representative p95 immunohistochemical images are shown in Fig. 4A and enlarged images in Supplementary Fig. S1. The results from the p95 VeraTag assay and p95 immunohistochemical assay were in broad agreement, although within each immunohistochemical category, there was a range of values from the p95 VeraTag assay (Fig. 4B). The immunohistochemical analysis using a semiquantitative H-score showed a correlation (R² = 0.39; P < 10⁻¹⁵) between these two methods of quantifying p95 (Fig. 4C). More samples were below the limit of detection in the immunohistochemical format (29%) than in the VeraTag p95 format (4.6%).

As with the p95 VeraTag analysis, the p95 immunohistochemical analysis was focused on the cases that were HER2 positive by VeraTag. Kaplan–Meier plots for the

<table>
<thead>
<tr>
<th>Table 2. Patient outcomes according to VeraTag p95 status in VeraTag HER-2-positive patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Category</strong></td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Univariate analysis</strong></td>
</tr>
<tr>
<td>All VeraTag HER2 positive (n = 190)</td>
</tr>
<tr>
<td>VeraTag p95 positive</td>
</tr>
<tr>
<td>VeraTag p95 negative</td>
</tr>
<tr>
<td>Hormone receptor negative (n = 112)</td>
</tr>
<tr>
<td>VeraTag p95 positive</td>
</tr>
<tr>
<td>VeraTag p95 negative</td>
</tr>
<tr>
<td>Hormone receptor positive (n = 78)</td>
</tr>
<tr>
<td>VeraTag p95 positive</td>
</tr>
<tr>
<td>VeraTag p95 negative</td>
</tr>
<tr>
<td><strong>Multivariate analysis</strong></td>
</tr>
<tr>
<td>VeraTag p95 positive</td>
</tr>
<tr>
<td>Hormone receptor positive</td>
</tr>
<tr>
<td>HER2 FISH positive</td>
</tr>
<tr>
<td>Grade 3</td>
</tr>
<tr>
<td>Stage IV</td>
</tr>
<tr>
<td>Ductal</td>
</tr>
<tr>
<td>Age &gt; 55</td>
</tr>
</tbody>
</table>

NOTE: Hormone receptor positivity defined as either ER-α or PR positivity (≥10% positive nuclear staining). Univariate results including all patients were stratified by hormone receptor status and tumor grade. Hormone receptor subset analyses were stratified by tumor grade.

Abbreviation: NR, not reached.

*p VeraTag HER2 positive and p95 positive defined as H2T > 13.8 RF/mm² and p95 ≥ 2.8 RF/mm², respectively.
p95 immunohistochemical categories are shown in Supplementary Fig. S2A and S2B. In contrast with the p95 VeraTag results, the p95 immunohistochemical categories did not correlate with either PFS \((P = 0.93)\) or OS \((P = 0.54)\). Cutoffs between each immunohistochemical level were also tested, but none gave a statistically significant result for PFS or OS. Continuous p95 H-score and IHC 0/1+ versus 3+ were also tested with the same result.

With the VeraTag p95 assay, the strongest correlations with PFS and OS were seen in the hormone receptor-positive subset. P95 IHC in this group showed a trend with shorter PFS \((P = 0.071)\) and no correlation with OS \((P = 0.96; \text{Supplementary Fig. S2C})\).

**Discussion**

Since the discovery of a truncated form of HER2 as a potential biomarker of trastuzumab resistance, the molecule commonly called p95HER2 or p95 has been measured and defined in a number of different ways, each capturing a different subset of the mixture of active and inactive members of the HER2-CTFs family \((4)\). Not surprisingly, attempts to infer active p95 levels by comparing HER2 intracellular and extracellular domain content have led to inconsistent results \((13)\). Even if the expression of various HER2-CTFs were correlated to some degree, obtaining an accurate measure of the minor component of active HER2-CTF would be plagued by a high degree of variability. This situation prompted the development of specific antibodies for the M611-HER2-CTF \((6, 8)\).

Using the M611-HER2-CTF–specific D9 antibody in a VeraTag assay, an optimal clinical cutoff for poor outcome in trastuzumab-treated MBC patients was established in a training set \((6)\) and verified in the current test set. As a marker for poor outcome of trastuzumab, high p95 expression may identify tumors that are susceptible to a HER2 tyrosine kinase inhibitor (TKI) used alone or in combination with trastuzumab-containing therapy \((14)\). Upcoming
clinical studies comparing trastuzumab with trastuzumab in addition to a HER2 TKI, with prospective p95 assessment, may verify this hypothesis.

In this study, surprisingly the hormone receptor-positive cases seemed to be the major driver of the correlation between p95 and outcomes. This may be unique to the metastatic setting, where most patients with hormone receptor-positive disease had already progressed on endocrine therapy in the adjuvant setting. Some of this resistance to endocrine therapy may have been driven by p95. The observed relationship between p95 levels and hormone receptor status is consistent with a previous finding (7). The reasons why the hormone receptor-positive set would drive this correlation are not well understood, as the characteristics seemed to be well distributed between hormone receptor-negative and -positive groups (Table 1) except for a higher likelihood for hormone receptor-negative tumors to be grade 3 ($P = 0.0056$). A recent report has revealed that p95 expression can induce a senescence secretory phenotype that can support metastasis of proliferating breast cancer cells (5). It could be hypothesized that with continued p95 expression in metastatic lesions, as we have observed (15), p95-induced protumorigenic factors may promote a more aggressive phenotype. However, it is unclear why this would be enhanced in the hormone receptor-positive subset.

At first glance, the percentage of HER2-positive tumors with p95 expression above the prespecified p95 cutoff may seem higher than expected. However, earlier reports on rates of p95 positivity were based an analytical cutoffs that may have been limited by the sensitivity of the methods used (1, 2). As shown in Fig. 2, there is no obvious analytical break point that could be used to define p95 positivity. The p95 VeraTag cutoff was set in the training set (6) based on clinical outcomes. The training set had a lower percentage of cases above the p95 cutoff, but was also composed of a population with lower H2T and FISH HER2/CEP17 distributions.

VeraTag was chosen as the preferred format due to increased sensitivity, quantitative output, and to avoid the subjective quantification that is required for IHC. Other quantitative approaches may give a readout more similar to VeraTag, such as visualization of immunofluorescence directly on the slide versus capillary electrophoresis quantification of the cleaved fluorescent tag used in the VeraTag method. IHC was chosen here as a point of comparison because it is the most commonly used method for assessing protein expression in FFPE samples. The p95 immunohistochemical assay was optimized for specific p95 detection in the presence of HER2 and maximal staining in positive cases without appreciable background in negative cases. Although it is conceivable that a different staining protocol could have yielded somewhat different results, we believe that the data presented here are a valid comparison of p95 by VeraTag and p95 IHC. In the current cohort, there was a broad correlation between VeraTag assay and IHC using the same antibody (Fig. 4C; $R^2 = 0.39$; $P < 10^{-15}$); however, IHC did not distinguish favorable from poor outcomes. Some of this variation likely comes from the method of quantification. As is customary with IHC scoring, the immunohistochemical category was determined by the staining level of the most intensely stained cells, in this case the top 10%. In contrast, the VeraTag assay provides a measure of the tumor averaged p95 expression. Therefore, more heterogeneous staining could give a higher immunohistochemical category than homogeneous staining at the same p95 VeraTag level. Nonetheless, IHC may still prove useful in detecting heterogeneous expression of p95 within the tumor, should this prove to be important. The correlation of treatment efficacy outcomes with p95 VeraTag values and the lack of correlation with p95 IHC suggest that a tumor-averaged measurement of p95 expression using the VeraTag method may be more clinically useful.
Recently, a different p95 immunohistochemical assay was assessed in patients administered neoadjuvant trastuzumab in the GeparQuattro study (16). This assay used a different antibody and at a concentration high enough to give 82% p95 positivity, using a cutoff of 20% strong staining, among those that were HER2 positive by silver in situ hybridization. Surprisingly, p95 positivity at this cutoff correlated with a higher pathologic complete response (pCR) rate. It is not known whether inadvertent measurement of full-length HER2 or a difference in setting contributed to this result. In support of the former point, HER2 mRNA levels have been found to correlate with increasing pCR in GeparQuattro at least in the ESR1-positive tumors (17). A recent report of the VeraTag p95 assay in the NeoALITTO clinical trial (18) also supports the notion that p95 expression in the neoadjuvant setting may have different significance than in the metastatic setting investigated here. Although pCR generally correlates with longer term outcomes, this is not the case in luminal B/HER2-positive-like tumors (19), the subgroup whose outcomes were most correlated with p95 expression in the current metastatic study.

Even though our study is among the few examples of positive validation of a novel predictive marker in advanced breast cancer, we are aware of its limitations. First, it was retrospective, although the p95 cutoff was prespecified. Second, this study used measurements on primary tumors to predict outcomes in the metastatic setting, whereas some tumor characteristics might have changed during progression. We have compared the VeraTag assay with p95 IHC rather than with a more quantitative method such as immunofluorescence that might yield similar results to VeraTag. Finally, as with any tissue-based immunoassay, preanalytic sample processing might have impacted measurements in FFPE tissue. Some of these factors may have resulted in the relatively modest PFS difference observed in the p95 subgroups (HR = 1.43). The difference was greater in the training set (HR = 1.9; ref. 6), as expected for an optimized cutoff. The OS results were more similar in the current (HR = 1.94) and training (HR = 2.2; ref. 6) sets.

In conclusion, a clinical VeraTag p95 cutoff derived from a previous training dataset was confirmed in a second independent clinical series as a predictor of clinical outcomes on trastuzumab. The observed consistency in the p95 VeraTag cutoff across different cohorts of patients with MBC treated with trastuzumab justifies additional studies using blinded analyses in larger series of patients. Clinical relevance of quantitative p95 protein expression remains to be established in a controlled clinical trial.

Disclosure of Potential Conflicts of Interest
J. Sperinde is employed as a director/scientist and has ownership interest (including patents) in LabCorp. A. Chenna is employed as a scientist and has ownership interest (including patents) in LabCorp. A. Paquet has ownership interest (including patents) in Labcorp stock. J.M. Weidler is employed in Monogram Biosciences/LabCorp. W. Huang is employed as a senior director, Clinical Research in Monogram Biosciences/Integrated Oncology-LabCorp. J. Winslow is employed as director, Oncology in Monogram Biosciences/LabCorp. No potential conflicts of interest were disclosed by the other authors.

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Received October 9, 2013; revised February 17, 2014; accepted February 17, 2014; published OnlineFirst March 25, 2014.

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Clinical Cancer Research
OF8
Clin Cancer Res; 20(10) May 15, 2014


Clinical Cancer Research

Quantitative Measurements of Tumoral p95HER2 Protein Expression in Metastatic Breast Cancer Patients Treated with Trastuzumab: Independent Validation of the p95HER2 Clinical Cutoff

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Clin Cancer Res  Published OnlineFirst March 25, 2014.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-13-2782

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