Quantitation of p95HER2 in Paraffin Sections by Using a p95-Specific Antibody and Correlation with Outcome in a Cohort of Trastuzumab-Treated Breast Cancer Patients

Jeff Sperinde1, Xueguang Jin1, Jayee Banerjee1, Elicia Penuel1, Anasuya Saha1, Gundo Diedrich2, Weidong Huang1, Kim Leitzel3, Jodi Weidler1, Suhail M. Ali3,4, Eva-Maria Fuchs5, Christian F. Singer6, Wolfgang J. Köstler5, Michael Bates1, Gordon Parry1, John Winslow1, and Allan Lipton3

Abstract

Purpose: p95HER2 is an NH2-terminally truncated form of HER2 that lacks the trastuzumab binding site and is therefore thought to confer resistance to trastuzumab treatment. In this report, we introduce a new antibody that has enabled the first direct quantitative measurement of p95HER2 in formalin-fixed paraffin-embedded (FFPE) breast cancer tissues. We sought to show that quantitative p95HER2 levels would correlate with outcome in trastuzumab-treated HER2-positive metastatic breast cancer.

Experimental Design: The novel p95HER2 antibody used here was characterized for sensitivity, specificity, and selectivity over full-length HER2. Quantitative p95HER2 levels were measured in 93 metastatic breast tumors using a VeraTag FFPE assay to determine the correlation of p95HER2 levels with outcomes.

Results: Within a cohort of trastuzumab-treated metastatic breast cancer patients, high levels of p95HER2 were found to correlate with shorter progression-free survival [hazard ratio (HR), 1.9; P = 0.017] and overall survival (HR, 2.2; P = 0.012) in patients with tumors selected to be HER2 positive by the VeraTag HER2 assay. For those with tumors found to be fluorescence in situ hybridization positive, elevated p95HER2 correlated similarly with shorter progression-free survival (HR, 1.8; P = 0.022) and overall survival (HR, 2.2; P = 0.009).

Conclusions: We have successfully generated an antibody that can specifically detect p95HER2, and developed an assay to quantify expression in FFPE tumor specimens. Using this novel assay, we have identified a group of HER2-positive patients expressing p95HER2 that have a worse outcome while on trastuzumab. As p95HER2 retains sensitivity to kinase inhibitors, measurement of p95HER2 in breast tumor sections may be useful in guiding treatment for patients with HER2-positive breast cancer.

HER2/ErbB2 is a member of the epidermal growth factor receptor family of tyrosine kinases that plays a significant role in a variety of cancers (1). Elevated expression of HER2 is found in 25% to 30% of breast cancers and is associated with poor prognosis (2, 3). Some portion of the prognostic significance of HER2 overexpression may be due to associated expression of p95HER2 (4), a truncated form of HER2 lacking the extracellular domain (ECD). p95HER2 is phosphorylated (5); readily forms homodimers (6) and heterodimers with HER3 (7), presumably due to the lack of ECD that can act as a negative regulator for dimerization (8); upregulates HER1 (9); and is associated with other negative prognostic factors, including nodal status (10).

p95HER2 was originally identified as the amino-terminally truncated fragment(s) of HER2, presumed to be the remnant of metalloprotease-mediated proteolytic cleavage of the HER2 ECD (5). More recently, multiple amino-terminally truncated fragments of HER2, or carboxy-terminal fragments (HER2-CTF), have been identified in human breast tumors that show similar but distinct migration patterns on Western blots probed with antibodies to the intracellular domain (ICD) of HER2 (6, 10–12). A number of these additional HER2-CTFs are likely the product of translation of the HER2 gene initiated at alternate AUG codons (12). Recently, the HER2-CTF starting at methionine 611 of the HER2 gene, M611-HER2-CTF, was found to be

Authors’ Affiliations: 1Monogram Biosciences; 2Diadexus, South San Francisco, California; 3Division of Hematology-Medical Oncology, Penn State/Hershey Medical Center, Hershey, Pennsylvania; 4Lebanon VA Medical Center, Lebanon, Pennsylvania; and 5Division of Oncology, Department of Medicine I and 6Department of Obstetrics and Gynecology, Medical University of Vienna, Vienna, Austria

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Corresponding Authors: John Winslow, Monogram Biosciences, 345 Oyster Point Boulevard, South San Francisco, CA 94080. Phone: 650-6244543. Fax: 650-6244132. E-mail: jwinslow@monogrambio.com and Allan Lipton, Section of Hematology-Oncology, Penn State Hershey Medical Center, 500 University Drive, Hershey, PA 17033. E-mail: alipton@hmc.psu.edu.

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most potent in inducing signal transduction pathways (6). Another HER2-CTF, A648-HER2-CTF, presumed to be one of the products of HER2 sheddase activity (13), was also found to be active, albeit to a lesser extent than M611-HER2-CTF (6). In the current study, we will maintain the presence of p95 as the family of HER2-CTFs with relative migrations by Western blot of M, ~ 95,000, although particular interest will be given to the highly active M611-HER2-CTF form, which is often referred to as p95HER2 (14, 15).

p95 has often been cited as a likely determinant of trastuzumab resistance (16) because it lacks the HER2 ECD trastuzumab binding domain. This may be mitigated at least in part by the ability of trastuzumab to inhibit HER2 sheddase activity by binding near the putative cleavage site (11). Overall, high p95 expression may be a net negative indicator of response to trastuzumab, as suggested by data from a small cohort of metastatic breast cancer patients (14), although the abundance of inactive non–membrane-bound truncated HER2 (6) may have influenced the type of p95 measurement used in this report. Quite separate from the question of trastuzumab resistance, p95 activity is sensitive to antagonism by HER2-directed tyrosine kinase inhibitors, a quantitative measurement of p95 may be useful in guiding treatment decisions for patients with HER2-positive breast cancer.

Translational Relevance

p95, an NH₂-terminally truncated form of HER2 that lacks the trastuzumab-binding site, is often cited as a contributing cause of trastuzumab resistance. Currently, p95 cannot be directly measured in formalin-fixed, paraffin-embedded (FFPE) tissues, although some indirect methods have been developed from which the presence of p95 may be inferred. Here, we characterize a novel p95 antibody in cell lines and tumors that displays high selectivity over full-length HER2, and present a quantitative p95 assay for the analysis of FFPE clinical tissue samples. We further used this assay to identify a subgroup of high p95 expression with less favorable outcome in a cohort of HER2-positive metastatic breast cancer patients who received trastuzumab-containing therapy. Because p95 retains the HER2 tyrosine kinase domain and is sensitive to kinase inhibitors, a quantitative measurement of p95 may be useful in guiding treatment for patients with HER2-positive breast cancer.

Immunizations and antibody production

Peptide D (MPIWKFPDEEGAC, Biomatik) was conjugated to keyhole limpet hemocyanin for immunizations and bovine serum albumin (BSA) for screening. Immunizations and antibody production and purification were carried out as detailed in ref. (19). The anti-p95 clone D9 was identified by enzyme-linked immunosorbent assay (ELISA) and flow cytometry screening.

ELISA

HER2-ECD-Fc and HER3-ECD-Fc were purchased from R&D Systems. Peptide A (ASPLTSIIS, Biomatik), peptide D, HER2-ECD-Fc, and HER3-ECD-Fc in PBS were adsorbed to ELISA plates overnight at 4°C in triplicate. Plates were blocked with 3% BSA (Sigma) in PBS containing 0.05% Tween 20 (PBSTw) for 1 hour. Following two PBSTw washes, wells were probed with 1.0 μg/mL of either D9 or anti-HER2 Ab5 (Thermo Scientific) in PBSTw containing 3% BSA for 30 minutes. Following three washes with PBSTw, bound antibody was detected with goat anti-mouse horseradish peroxidase. The plates were developed using 1-Step TMB (Thermo Scientific) according to the manufacturer’s recommendations.

Expression vectors

Expression vectors for p95 and full-length HER2 were constructed using the pcDNA6A-myc/His vector from Invitrogen. The HER2 expression sequence included a hemagglutinin (HA) tag (YPYDVPDYA) two amino acids downstream of the putative leader sequence cut site. A stop codon was included at the end of the HER2 sequence to prevent the incorporation of the myc/His tags embedded in
The p95 sequence began with Met611, numbered from the HER2 amino acid sequence. Upstream from Met611 was placed a sequence encoding the HER2 leader sequence plus two amino acids followed by the same nine-amino-acid HA tag used in the HER2 expression vector.

**Western blots**

Samples were mixed with 4× Laemmli buffer (Bio-Rad) and heated to 70°C for 10 minutes. Separately, protein content was assessed by bicinchoninic acid assay (Pierce) according to the manufacturer's instructions. Samples of equal protein content were loaded into a 4% to 12% gradient gel and run in a MOPS-based running buffer (Invitrogen). Bands were transferred to a nitrocellulose membrane (Invitrogen) in Nu-PAGE transfer buffer (Invitrogen) + 10% methanol. The membrane blots were first blocked with 1% Triton X-100 in PBS plus 3% nonfat dry milk. With washes with 1% Triton X-100 in PBS before each step, blots were stained with anti-HER2 Ab8 (Thermo Scientific) then goat anti-mouse linked with horseradish peroxidase (Pierce) and developed with the West Dura horseradish peroxidase detection kit (Pierce) according to the manufacturer's instructions. Densitometry was done using ImageJ (20).

**Flow cytometry**

293F cells were transfected with p95 or HER2 expression vectors using 293-fectin (Invitrogen) according to the manufacturer's instructions. After 2 days, the cells were fixed with paraformaldehyde. Anti-ricin (negative control), anti-HA (positive transfection control), or D9 were incubated with the cells. Bound antibodies were detected using a fluorescein-conjugated anti-mouse IgG.

**FFPE blocks from cell lines**

FFPE blocks from cell lines were produced as in ref. (18). Cells intended for transient transfection were allowed to attach for 4 hours and were then transfected 2 days before fixation with Fugene HD (Roche) according to the manufacturer's instructions.

Cell lysates were prepared from samples of cells removed and lysed just before the addition of neutral buffered formalin (Richard-Allan) described in ref. (18). Lysis buffer contained 1% Triton X-100, 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 50 mmol/L NaF, 50 mmol/L sodium β-glycerophosphate, 1 mmol/L Na3VO4, 5 mmol/L EDTA, and one tablet of Complete Protease Inhibitor (Roche 1836170) in 10 mL water.

**HER2 VeraTag assay**

The HER2 VeraTag assay was done as described in refs. (18, 21, 22) using anti-HER2 ICD antibodies Ab8 and Ab15 (Thermo Scientific).

**p95 VeraTag assay**

Deparaffinization and antigen retrieval was done as in ref. (18), except that antigen retrieval was done in a pressure cooker (Decloaking Chamber, Biocare Medical) using Diva buffer (Biocare Medical). Samples were blocked with 10% goat serum (Sigma) and 1.5% BSA in PBS (blocking buffer). Slides were stained with 4 μg/mL anti-p95 D9 and then with 1 μg/mL goat anti-mouse antibody (Thermo Scientific) labeled with a fluorescent VeraTag (18), with washes between each step using PBS containing 0.25% Triton X-100. Slides were washed with PBS/Triton X-100 and then with deionized water. Residual liquid was removed using a centrifuge (Tomy PMC-082) modified to spin slowly. Following centrifugation, 200 μL capture buffer containing 1.0 mmol/L dithiothreitol, 3 pmol/L fluorescein, and two electrophoresis internal markers in 0.01× PBS was added to release the VeraTags by reduction. Quantification of released VeraTags was achieved by capillary electrophoresis. Capillary electrophoresis injection parameters, fluorescent peak quantification, and tumor area assessment by a pathologist was done as detailed in ref. (18). The final value for p95 content was calculated as [relative fluorescence (RF) concentration] × [capture buffer volume] / [tumor area] giving final units of RF/mm² tumor. Normalization between independent experiments, typically ~15 slides per batch, is achieved by aligning cell line controls, MCF7, SKBR3, MCF-p95 clone A, and MCF7-p95 clone B, to predetermined values by a least-squares fit.

**Peptide competition**

For peptide competition experiments, the VeraTag p95 assay was done as usual except that peptides D or Dsc (KCWAEDGFPEPMI, Biomatik) were incubated with the D9 antibody for 15 minutes at the indicated concentration before electrohoresis. Capillary electrophoresis injection parameters, fluorescent peak quantification, and tumor area assessment by a pathologist was done as detailed in ref. (18). The final value for p95 content was calculated as [relative fluorescence (RF) concentration] × [capture buffer volume] / [tumor area] giving final units of RF/mm² tumor. Normalization between independent experiments, typically ~15 slides per batch, is achieved by aligning cell line controls, MCF7, SKBR3, MCF-p95 clone A, and MCF7-p95 clone B, to predetermined values by a least-squares fit.

**Immunohistochemistry**

 Vectastain ELITE ABC Peroxidase kit, ImmPact 3,3'-diaminobenzidine peroxidase substrate, and hematoxylin were all from Vector Laboratories. The epitope retrieval process was identical to that described for the VeraTag p95 assay above. Following the 1-hour cooling step, sections were washed six times with water then blocked, stained with anti-p95 D9 (4 μg/mL) or anti-HER2 CB11 (1 μg/mL), and developed according to the recommended protocol for the ABC kit. Cell micrographs were taken with a digital camera image capture system mounted on a Leica microscope.

**Results**

**Anti-p95 antibody identified by ELISA**

Immunizations were done using a series of peptides comprising the extracellular region of p95 from HER2 Met611 to Ser656. Antibodies from individual clones were screened by peptide and protein assays to identify antibodies that bind p95 but not full-length HER2.

In an ELISA primary screen, antibodies were tested for binding to the immunization peptide, a control peptide, a recombinant human HER2 ECD-Fc fusion protein, and a recombinant human HER3 ECD-Fc fusion protein. D9, an antibody specific for p95, was identified in the series of
immunizations using peptide D (Met611-Cys623). Figure 1A shows the ELISA results using D9 compared with a commercially available anti-HER2 antibody, Ab5 (Thermo Scientific). As expected, the Ab5 antibody bound only HER2 ECD-Fc. In contrast, D9 specifically bound the D peptide used in immunizations, yet showed no significant binding to HER2-ECD-Fc although the primary sequence of peptide D is found in the HER2-Fc fusion protein (Met1-Thr652). This may indicate that D9 binds to an epitope on p95 that is inaccessible in the folded HER2-ECD.

Selectivity for p95 over full-length HER2 in fixed cells

As a secondary screen, antibodies were tested for the ability to bind full-length HER2 or p95 when expressed in 293F cells. Cells were transfected with vectors expressing either full-length HER2 or p95. Both expression vectors contained an NH2-terminal HA tag that enabled specific detection of the recombinant protein. A Western blot (Fig. 1B) shows the expression of both vectors in 293F cells as detected by Ab8 (Thermo Scientific), directed against the ICD of HER2. Consistent with previously published results, a small amount of p95 was found when recombinant HER2 was overexpressed presumably due to initiation of translation at an alternate methionine AUG (12). Figure 1 (C and D) shows flow cytometry results using fixed 293-HA-p95 and 293-HA-HER2 cells, respectively. In the 293-HA-p95 cells, D9 seemed to bind to p95 to a similar extent as the anti-HA antibody relative to an irrelevant anti-ricin antibody, consistent with specific binding of D9 to recombinant p95. In contrast, D9 showed only a minor shift from the anti-ricin antibody in the 293-HA-HER2 cells, binding to a far lesser extent than the anti-HA positive control antibody.

Selectivity of p95 over full-length HER2 in FFPE sections

FFPE MCF7 cells expressing full-length HER2 or p95 were used to assess p95 selectivity in the FFPE format by a VeraTag p95 assay using D9. MCF7 cells were transiently transfected with empty vector or the same p95 and HER2 expression vectors used above for production of both cell lysates and cell pellets to be made into FFPE blocks for sectioning. Cell lysates were analyzed by HER2 ICD Western blot (Fig. 2A) for comparison with VeraTag FFPE assay results. Some p95 expression in the MCF7-HER2 cells was detected as was noted above in 293-HA-HER2 cells. The VeraTag HER2 FFPE assay (18) was used to quantify the relative levels of HER2 ICD in the FFPE sections of...
MCF7, MCF7-p95, and MCF7-HER2 (Fig. 2B). The VeraTag p95 FFPE assay was then performed on sections from these same blocks to evaluate selectivity for p95 over HER2 in the FFPE format. The VeraTag p95 signal of the MCF7-p95 sections was well above that of the MCF7 sections (Fig. 2C). In contrast, the p95 signal from the MCF7-HER2 sections was only slightly higher than MCF7 despite the robust HER2 expression shown in Fig. 2B. The small amount of p95 signal detected from the MCF7-HER2 sections was consistent with the level of p95 detected by Western blot.

Concordance of VeraTag p95 measurement in FFPE cell lines with Western blot

Wild-type cell lines and p95-transfected MCF7 cells were used to confirm that the signals measured in the FFPE p95 assay were generally proportional to the amount of p95 found by Western blot. By HER2 Western blot, a panel of cell lines was shown to express various amounts of truncated HER2, as shown in Fig. 3A. MCF7 cells transfected with two levels of p95 (lanes 3 and 4) can be used to localize the HER2-CTF form of interest. When the p95 construct is highly overexpressed, MCF7-p95 cells show three bands in the 75 to 100 kDa range by HER2 Western blot. The three bands in increasing mobility are most likely glycosylated p95 (top arrow, Fig. 3A), nonglycosylated p95 (bottom arrow, Fig. 3A), and HER2-CTF starting at Met687 (6). Met687-HER2-CTF does not contain the epitope for the p95 antibody. Unfortunately, D9 is not functional in the Western blot format so it could not be used to rule out bands not containing the D9 epitope. However, another antibody raised against the same immunogen detects the upper two bands but does not detect the lowest molecular weight band (data not shown), consistent with the supposition that this lower band is Met687-HER2-CTF. Therefore, 

Fig. 2. Specificity of antibody D9 for p95 over HER2 in the VeraTag p95 assay. A, transient expression of p95, HER2, or empty vector assessed by HER2 ICD (Ab8) Western blot. The MCF-vector-only sample was run on the same gel as MCF7-p95 and MCF7-HER2, but in a nonadjacent lane. FFPE blocks were prepared from MCF7 cells transiently transfected with empty vector, p95, or HER2. Slides from these blocks were assayed using the p95 VeraTag assay (C) and HER2 ICD using the HER2 VeraTag assay (B), which detects both p95 and HER2.

Fig. 3. VeraTag p95 accuracy and specificity in FFPE cell lines. A, cell lines were made into lysates for HER2-ICD (Ab8) Western analysis and FFPE blocks for VeraTag p95 measurements. Densitometry was done on the two p95 bands indicated by arrows. B, VeraTag p95 measurements were plotted versus densitometry results from A ($R^2 = 0.94$). C, specific peptide (peptide D, solid symbols) or scrambled peptide (peptide Dsc, open symbols) was added to D9 before the assay for HCC1419 (diamonds) or MCF7 (squares) at the indicated concentrations. p95 values relative to no peptide MCF7 are shown as mean ± SE of duplicate measurements.
it is expected that the p95 signal obtained in the VeraTag p95 assay should be predominantly dependent on the bands indicated by the two arrows, although contributions from higher molecular weight fragments cannot be ruled out. Densitometry was done on the two bands indicated by arrows in Fig. 3A for comparison with VeraTag p95 values, measured in the FFPE sections made from these same cell preparations. In Fig. 3B, VeraTag p95 values were plotted against densitometry results from above. p95 levels, as measured by the VeraTag assay, correlated with the density of the two bands indicated by the arrows ($R^2 = 0.94$) within the limits of FFPE block packing variability between cell lines.

**Specificity of VeraTag p95 measurement in FFPE cell lines**

Peptide competition experiments were conducted to evaluate the specificity of the p95 antibody in the FFPE format. A p95-high cell line, HCC1419, and a p95-low/negative cell line, MCF7, were selected for competition experiments. The VeraTag p95 assay was done on FFPE slides from each cell line with increasing amounts of either specific peptide, peptide D, or scrambled peptide, peptide Dsc. The results shown in Fig. 3C show that the high p95 signal measured in HCC1419 cells was effectively competed away by the specific peptide but not the scrambled peptide. In contrast, for MCF7 cells, there was only a small displacement at the highest concentration tested by the specific peptide near the level of expected variation.

**Measurement of p95 in FFPE tumors**

To assess the reproducibility of the VeraTag p95 measurement in tumor sections, a series of 112 breast tumors were measured for p95 in duplicate. Fifteen of these were selected for high HER2 expression [immunohistochemistry...
(IHC) 3+ to ensure adequate representation across a range of HER2 expression. The remainder of the set represented a random sampling of HER2 levels. The reproducibility of the VeraTag p95 assay is represented in Fig. 4A by an iso-plot of duplicate p95 measurements ($R^2 = 0.97$). The variability was nearly constant across the entire dynamic range with a mean coefficient of variation of 11%.

Staining characteristics of D9 in FFPE breast cancer tissue were assessed by IHC. Although IHC is not as sensitive or quantitative as the VeraTag technology, IHC was done to give visual verification that the p95 antibody was providing primarily membrane staining of tumor cells. Two HER2-positive (IHC 3+) breast tumors were selected for comparison with high and low VeraTag p95 expression, respectively. The p95 staining in the high p95 tumor was found to be primarily membranous as expected, whereas only limited staining was seen in the low p95 tumor (Fig. 4B). In a separate experiment, D9 IHC was done on tumor B with or without competing peptide D. As seen in Fig. 4B, a high level of competition was observed similar to that seen in Fig. 3C. These IHC images also provide an illustration of the degree to which HER2-positive breast tumors may express very different levels of p95 that are largely independent of the level of HER2 expression.

**Measurement of p95 in FFPE tumor sections from a metastatic breast cancer cohort**

It has been suggested that p95 expression may cause trastuzumab resistance. To test whether the current p95 assay can be used to predict different outcomes for patients receiving trastuzumab, 93 tumors from a cohort of trastuzumab-treated metastatic breast cancer patients were measured for HER2 and p95 using VeraTag assays. Clinical characteristics and HER2 measurements for the current cohort were described previously (23). The VeraTag p95 assay was done on the FFPE tumor sections in eight batches. The performance of the cell line interbatch normalization controls is shown in Fig. 4C. The close alignment of the normalization controls indicates that the assay performed consistently across the multiple batches.

The distribution of p95 expression in this cohort as measured by the VeraTag assay was found to span approximately 10- to 20-fold, as shown in Fig. 4D. To define the sensitivity limit for the p95 assay in tumors, a mock assay was done on a set of 76 breast tumors where the specific p95 antibody, D9, was substituted with its isotype control. A sensitivity cutoff was set at a level above 95% of the isotype control measurements. Seventy-two percent of p95 signals from the tumors in the trastuzumab-treated cohort were found to be above this sensitivity cutoff. When considering only the tumors with overexpressed HER2 using the VeraTag HER2 assay (21–24) or those that were HER2 fluorescence in situ hybridization (FISH) amplified, 81% and 79%, respectively, of the p95 signals fell above this sensitivity limit.

Correlation of p95 expression with HER2 expression is shown in Fig. 5A. As expected, elevated p95 expression...
was rarely seen in the absence of HER2 overexpression or high HER2 FISH copy number (Fig. 5B). However, the level of HER2 overexpression was not always associated with high p95 levels. This is seen in Fig. 5A, where a weak correlation between p95 and HER2 was observed ($R^2 = 0.11$).

**Correlation of p95 expression with clinical outcomes**

High HER2 expression, as measured by the VeraTag HER2 assay, and high FISH copy number (HER2 FISH/CEP17 > 2.2) were previously shown to be significantly correlated with longer progression-free survival (PFS) following trastuzumab treatment in this cohort (23). Here, we sought to test the hypothesis that p95 measurements could be used to define subgroups of patients with different outcomes within the HER2-overexpressed or HER2 FISH-amplified subgroups. The cutoff for p95 was optimized to maximize the hazard ratio (HR) between patients with tumor p95 expression above versus below the cutoff within the HER2-positive subgroup. This cutoff was used along with the previously defined HER2 cutoff (22, 23) to define three groups: low HER2, high HER2 with low p95, and high HER2 with high p95. PFS and overall survival (OS) Kaplan-Meier plots for these three groups are shown in Fig. 5C and D, respectively. Statistics for these plots are given in Table 1. Within the high HER2 subgroup, those with p95 expression above the cutoff had significantly shorter PFS and OS than those with levels of p95 below the cutoff. A similar comparison was made using the same p95 cutoff within the FISH-positive population, using the three groups of FISH/CEP17 ≤ 2.2, FISH/CEP17 > 2.2 with low p95, and FISH/CEP17 > 2.2 with high p95. Similar to the HER2-overexpressed subgroup, within the FISH-positive subgroup, those with p95 expression above the cutoff had shorter PFS and OS than those with levels of p95 below the cutoff as shown in Table 1. The low HER2 subgroup also had a poor outcome, likely influenced by the HER2-negative and hormone receptor-negative (triple-negative) patients (25).

In a recent report, transgenic mice expressing M611-HER2-CTF were more than twice as likely to acquire detectable lung metastases as transgenic mice expressing full-length HER2 (6). We examined the current cohort to determine whether a similar phenomenon was observable in this cohort of human metastatic breast cancer patients. We also performed a similar analysis for a number of other common metastatic sites. The relative proportion of patients above or below the p95 cutoff for each metastatic site is summarized in Supplementary Table S1. In this cohort, those patients with tumors expressing p95 at levels above the cutoff were twice as likely to have lung metastases as those below the p95 cutoff ($P = 0.015$, Fisher’s exact test). Other possible trends were observed, but none reached the level of significance at $P < 0.05$.

**Discussion**

The clinical significance of HER2-CTFs was originally established using the correlation of clinical factors with the presence of a cluster of bands of $M_i \sim 95,000$ on a HER2-ICD Western blot (5, 10). Subsequently, the two predominant mechanisms of protease activity (26, 27) and de novo synthesis from alternative translational start sites (12) have been elucidated, which could result in multiple HER2-CTFs of varying proportions and activities.

To our knowledge, there has been only one published report where sequencing was used to identify the NH2 terminus of p95 resulting from protease activity by inference from COOH-terminal sequencing and mass spectrometry of shed ECD in SKBR3 culture supernatant (13). The COOH-terminal sequencing results were noted as being suggestive of multiple cut sites, with mixed sequence identifiable for the first COOH-terminal residue (R/N/A) and fourth COOH-terminal residue (A/G/Q) only. The most likely pairing of A(43%)-X-X-R(43%) was consistent with a minor peak found by mass spectrometry and would correspond to a HER2-CTF NH2 terminus at A648. However, other pairings of the possible first and fourth residues are consistent with a total of 20 possible HER2 ECD cleavage sites, including cuts preceding C513, C587, or E645, which would also release the ECD because these sites are not protected by disulfide bonds. Cleavage at multiple sites could be the product of different proteases or even a single protease, as the primary HER2 sheddase, ADAM10 (27), has been shown to act near the cell membrane in the case of growth factor release or as far away as adjacent cells in the case of the ephrin A5 (28). Additionally, cleavage sites can be variable as the substrate recognition is largely driven by elements distal from the cleavage site (29).

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<th>Table 1. Kaplan-Meier statistical summary</th>
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<tr>
<td>HER2 status</td>
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<td>VeraTag HER2 &lt; 13.8</td>
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Cleavage at C587, E645, or A648 would be predicted to disrupt trastuzumab binding by releasing most or all of the HER2-trastuzumab points of contact (30). HER2-CTFs formed by cleavage at all of these sites except A648 would be detectable by the anti-p95 antibody D9 studied herein based on the sequence of the immunization peptide.

Similarly, the de novo alternative translational start mechanism is predicted to yield multiple HER2-CTFs, predominantly starting with M611 and M687 (12). Existence of these forms in breast tumors has been inferred from similar Western blot migration patterns comparing recombinant HER2-CTFs with breast tumor lysates (6, 12). In one report, signal transduction capability of recombinant M611 and M687 forms were compared along with the A648 form to mimic one of the products of HER2 sheddase activity. M611-HER2-CTF, the p95 form used in the current study, was shown to be the most potent inducer of phosphorylation of Erk1/2, Akt, Src, and other regulatory proteins, whereas A648-HER2-CTF was less potent (6). M687-HER2-CTF seemed to be inactive as expected because it lacks the transmembrane domain and therefore is not likely to dimerize or productively interact with other HER family members.

With multiple potential forms of varying quantities and signaling potencies, the question arises as to which form(s) of p95 are most correlated with adverse outcomes and to what degree are p95 assays sensitive to and specific for the most relevant forms. Currently, there are at least four means of measuring p95. HER2 ICD Western blot is the most direct, but this technique suffers from low availability of frozen tumor samples, is only semiquantitative, and may suffer from the imprecision caused by overlapping bands. Subtractive methods whereby conventional IHC is used to subtract density scores of HER ICD from scores of ECD to infer p95 content from this difference would likely be hampered by the predominant amount of inactive M687-HER2-CTF found in tumors (6). A novel immunofluorescence method for inferring the presence of p95 by intracellular localization of the cytoplasmic domain shows promise (14), but might also be influenced by the omnipresent M687-HER2-CTF. Finally, the assay studied here is quantitative, uses readily available FFPE sections, and is specific for the membrane-bound forms of HER2-CTF containing the M611 epitope.

The D9 antibody used in the current work was raised against an NH2-terminal peptide of M611-HER2-CTF, the most potent HER2-CTF for signal transduction (6). Additional study is necessary to identify the biological importance of A648-HER2-CTF in vivo, as it has been shown to be a less active membrane-bound HER2-CTF form in cell lines (6). D9 also seems to be only minimally reactive toward HER2 (Figs. 1, 2, 3A/B, 4B, and 5A), indicating that the D9 epitope on p95 is inaccessible in the folded HER2-ECD. D9 might bind to HER2Δ16 (31), an oncogenic splice variant of HER2, as the two missing cysteines might lead to misfolding that could expose the D9 epitope. This is currently under investigation. The D9 p95 VeraTag assay signal generally agrees with levels of naturally and recombinantly expressed cell line HER2-CTFs quantified by Western blot analysis and identified by relative gel mobility of recombinant M611-HER2-CTF (Figs. 2 and 3B). Importantly, D9 would not detect the inactive, non–membrane-bound HER2-CTFs such as M687-HER2-CTF. D9 would be predicted to detect most of the possible forms of HER2-CTFs produced by protease activity. However, A648-HER2-CTF should not be detectable as its sequence does not overlap with that of the immunization peptide used to generate D9. To the extent that A648-HER2-CTF levels correlate with trastuzumab resistance, high levels of A648-HER2-CTF could weaken the relationship between the D9 assay signal and outcome on trastuzumab. However, this will be mitigated to the degree that levels of other HER2-CTFs containing the D9 epitope correlate with A648-HER2-CTF.

Is this assay profile sufficient to identify trastuzumab-treated patients with poor outcomes? The results shown in Fig. 5 and Table 1 suggest that the D9-based VeraTag p95 assay can be used to identify patients with clinically important levels of p95. In a subset of patients confirmed to have HER2 overexpression by a VeraTag HER2 assay or FISH/CEP17 amplification, high VeraTag p95 values correlated with shorter PFS and OS. Future studies will include efforts to confirm these findings and to determine whether p95 measurements can be used to predict trastuzumab resistance. Because p95 is thought to be sensitive to HER2-active tyrosine kinase inhibitors such as lapatinib (14, 17) and HSP90 inhibitors (15), measurement of quantitative p95 levels may have potential in influencing treatment decisions.

Disclosure of Potential Conflicts of Interest

Allan Lipton: commercial research grant and expert testimony, Monogram Biosciences. All Monogram-affiliated authors are employees of Monogram Biosciences.

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