Validation of a Phosphoprotein Array Assay for Characterization of Human Tyrosine Kinase Receptor Downstream Signaling in Breast Cancer

Fadila Chergui,1,3† Anne-Sophie Chrétien,1,3† Sanae Bouali,1,3 Carole Ramacci,1,3 Marie Rouyer,1,3 Thierry Bastogne,4 Pascal Genin,2,3 Agnès Leroux,2,3 and Jean-Louis Merlin1,3*

BACKGROUND: Human epidermal growth factor receptor (HER) downstream signaling kinases have important effects on tumor response to anti-HER monoclonal antibodies and tyrosine kinase inhibitors. We validated an assay that uses phosphoprotein arrays for measurement of HER downstream signaling functionality in breast carcinomas.

METHODS: Using the Bio-Plex® phosphoprotein array (BPA), we performed multiplex immunoanalysis to investigate the expression of phosphorylated epidermal growth factor receptor and phosphorylated HER downstream signaling proteins (phosphorylated protein kinase B, phosphorylated glycogen synthase kinase –3β, phosphorylated P70 ribosomal protein S6 kinase, and phosphorylated extracellular signal regulated kinase 42/44) in 49 frozen specimens of ductal infiltrating breast carcinoma taken at diagnosis. BPA was cross-validated with Western blot analysis. Sample size, homogeneity, tumor content, protein extraction, and monoclonal antibody detection were in accordance with optimized standard operating procedures.

RESULTS: Linear regression showed significant quantitative correlations between BPA and Western blot, with regression coefficient values of 0.71–0.87 (P < 0.001). BPA intra- and interassay CVs were <17% and 15%, respectively. Compared to limits of detection established by using the mean + 3SD of 10 blanks, large variations of phosphoprotein expression, up to several hundred-fold, were observed among the 49 tumor specimens.

CONCLUSIONS: Our results validate the use of the multiplex phosphoprotein array assay in human clinical tumor specimens. Further prospective evaluation is warranted to investigate the use of HER downstream signaling phosphoproteins as predictive and/or surrogate markers for clinical response to anti-HER targeted therapy.

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The human epidermal growth factor receptor (HER) family has been extensively studied because of its key role in cancer biology (1). Activation of the HER receptors leads to the activation of several signal transduction pathways, among which the phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) and RAS/RAF/mitogen-activated protein kinase (MAPK) kinase pathways play a major role (2).

HER are overexpressed in many human malignancies, including breast cancer, head and neck squamous cell carcinoma, colon cancer, and lung cancer, and are associated with poor prognosis (3). Therefore, inhibition of HER has been investigated as a therapeutic strategy and has opened the way for so-called targeted therapies. In breast cancer, targeted therapy includes HER1- and HER2-directed monoclonal antibodies and tyrosine kinase inhibitors (4–6).

The PI3K/AKT signaling pathway is regulated by phosphatase and tensin homolog deleted on chromosome 10 (PTEN). Molecular defects of PTEN, which have been reported in breast cancer tumors as well as in other tumor types, lead to the constitutive phosphorylation of AKT and dramatically alter the cellular response to anti-HER drugs (7–11).

The RAS/RAF/MAPK signaling pathway is also involved in the cellular response to anti-HER drugs. Mu...
tations of genes associated with Ras and Raf occur frequently (approximately 50% of tumors) in several malignancies, such as colorectal and lung cancers (12), and occur to a lesser extent (approximately 5% of mutated tumors) in breast cancer (13). Retrospective studies (14, 15) have demonstrated the importance of documenting Ras mutational status in colorectal tumors, with Ras mutation leading to a dramatic reduction of response to cetuximab-based therapy and progression-free survival. Consequently, Ras genotyping has now been included in the prescription criteria of cetuximab and panitumumab in colorectal cancer patients. Recently, we demonstrated that analysis of the functionality of the Ras/RAF/MAPK signaling pathway could yield additional markers for identification of patients who have wild-type KRAS (the human homolog of the Kirsten rat sarcoma-2 virus oncogene) tumors but are still at risk for low progression-free survival (16).

Molecular diagnosis may be a useful tool in formulating strategies for alternative individualized therapy, as suggested by recent results achieved in gefitinib-resistant cells (17), which demonstrated that growth inhibition and apoptosis were restored by downstream blockade of the Ras/RAF/MAPK pathway by mitogen-activated extracellular signal regulated kinase–activating kinase (MEK).

These experimental results highlight the importance of examination of the functionality of selected proteins involved in the PI3K/AKT and Ras/RAF/MAPK signaling pathways in tumor tissues, because these proteins may be useful in predicting the response to anti-HER drugs (18). In the currently approved indications for breast cancer treatment, paradoxically, HER2 expression status remains the only molecular criterion currently evaluated to identify patients eligible for trastuzumab therapy (19).

Novel detection methods, similar to those recently adopted for KRAS mutation analysis in colorectal cancer, have been proposed to delineate the genetic and proteomic profiles of breast cancers. For example, a multiplex assay to measure the expression of PTEN, insulin-like growth factor receptors 1 and 2, and P95HER2/neu together with epidermal growth factor receptor (EGFR) and P185HER2/neu has been proposed to aid treatment decisions regarding trastuzumab and tyrosine kinase inhibitors (20).

Thus, exploring the functionality of HER downstream signaling could identify fingerprinting molecular defects that could impair the tumor response to HER-targeted therapies. The evaluation of the functionality of the signaling pathways can be assessed through the determination of multiple key phosphoproteins. In clinical studies the exploration of signaling phosphoprotein expression remains a technical challenge because only needle biopsies are available at diagnosis or during treatment, and these should be performed for multiple examinations. Development of an innovative technology allowing the determination of multiple key proteins is critical. The Bio-Plex® protein array (BPA) technology (Bio-Rad), which uses Luminex® beads and immunofluorescence, can be used for small clinical biopsies to evaluate the functionality of signal transduction pathways through the quantification of the expression of phosphorylated forms of multiple signaling proteins. A recent comparative study (21) established that the Luminex technology can be applied to detect multiple antigen–antibody reactions in patient samples in which these interactions are traditionally assessed individually by ELISA.

We validated the use of BPA for determination of multiplex signaling phosphoprotein in breast cancer clinical specimens before investigating the potency of signaling phosphoprotein expression for response prediction to anti-HER targeted therapy.

Patients, Materials, and Methods

Patients and Tumor Characteristics

Frozen tumor samples of primary breast cancer, obtained from 49 patients with infiltrating ductal carcinoma confirmed by immunohistochemical analysis, were obtained from our tumor bank, in accordance with the French regulations (bioethical law from August 6, 2004) and after we obtained approval of the study by the steering committee of the tumor bank. The median patient age at diagnosis was 56 years (range 28–93 years).

Breast cancer tissues, initially macroscopically selected by the pathologists, were obtained immediately after surgery (maximal ischemia time was 30–45 min from clamping of the vessels to freezing of the tumor specimens), shock frozen in liquid nitrogen, and then cryopreserved at −80 °C. Specimens were banked during the period 2006–2007.

Immunohistochemical analysis used to detect HER-2 overexpression revealed that tissue samples from 35% of the patients were HER2 positive and from 65% were HER2 negative, 74% of the tumors were SBR (Scarff Bloom Richardson) grade 3 and 26% were grade 2. Estrogen-receptor status determined by immunohistochemistry was positive in cancer tissue from 29 patients (63%) and negative in 20 (37%). Progesterone receptor status was positive in 14 (39%) and negative in 35 (61%). None of the patient sample donors had received any preoperative adjuvant hormone therapy or chemotherapy.

Frozen protein extracts from the HER1-overexpressing human breast cancer cell line (MCF7) exposed to EGF were used as positive controls.
PROTEIN EXTRACTION

We extracted proteins from cell cultures or tumor specimens by using a cell lysis kit (Bio-Rad) according to the manufacturer’s recommendations. Tumor specimens weighing 20 ± 5 mg were first disrupted for 15 min by use of a steel-bead tissue lyser (Quiagen).

Cell cultures and disrupted tumor tissues were exposed for 10 s to the lysis solution containing phenylmethylsulfonyl fluoride antiprotease. Proteins were stored frozen at −80 °C until analyzed.

WESTERN BLOT ANALYSIS

We used previously reported protocols, with slight modifications, to perform Western blots on protein extracts (22). The proteins were electrophoretized at 100 V for 150 min (PowerPac 200, Bio-Rad) in SDS-polyacrylamide gels (5%–10%) and then transferred onto a polyvinylidene difluoride membrane (Bio-Rad) at 80 mA for 30 min by use of a Transblot SD (Bio-Rad). Nonspecific binding was blocked with 5% milk in DMEM PBS/0.1% Tween 20 for 1 h at room temperature. Immunodetection of the proteins was performed with specific primary antibodies (Cell Signaling) directed against phosphorylated (p)-AKT, p–glycogen synthase kinase 3β (p–GSK3β), p–extracellular signal regulated kinase 42/44 (p–ERK1/2), and p–P38MAPK, within the dilution range 1/500–1/3000, and incubated overnight at 4 °C (Table 1). Tubulin was used as the internal loading control. The membranes were washed and incubated with horseradish peroxidase–secondary anti-IgG1 polyclonal antibodies (Cell Signaling). Immunoreactive proteins were visualized by using electrochemiluminescent reagent (Amer sham Biosciences).

Each dried blot was numerized at 100 dpi (Perfection 1670 scanner; Epson) and processed using ImageJ, Java-based image processing software (Rasband WS; NIH).

The area of the peak was outlined, and the proportion of the peak area as a percentage of the MCF7 positive control (for simplicity referred to as peak area) was used for statistical analysis.

BPA ASSAY

To analyze the expression of the signaling phosphoproteins, we used BPA (Bio-Rad) based on multiplex sandwich bead immunoassays previously used in breast, head and neck (18), and colorectal tumor specimens (16). Three independent technicians participated in the study. Protein extracts were transferred into 96-well dishes and diluted with 25.10⁻⁶ L buffered solution. Fluorescent capturing beads coupled to antibodies (Table 1) directed against p-AKT, p–GSK3β, p–P70 ribosomal protein S6 kinase (p–P70S6K), p–MEK1, p–ERK1/2, and p–P38MAPK phosphoproteins were mixed. p–EGFR expression was analyzed separately according to the manufacturer’s instructions. The antibody-conjugated beads were added into each well and incubated overnight. The plates were washed and incubated with biotinylated antibodies to fix each target protein. Streptavidin–phycoerythrin solution was then added. The analysis consisted of a double-laser fluorescence detection, which allowed simultaneous identification of the target protein through the red fluorescence emission signal of the bead and quantification of the target phosphoprotein through the fluorescence intensity of phycoerythrin. A positive control, consisting of a standard protein extract from control cell line, was added to each series. Results were recorded as mean fluorescence intensities and normalized to the data measured in the positive controls.

### Table 1. Phosphorylation-specific antibodies used in the Bio-Plex phosphoprotein array (BPA) and Western blot assays.

<table>
<thead>
<tr>
<th>Phosphoprotein</th>
<th>BPA antibody</th>
<th>Western blot antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-EGFR</td>
<td>Tyr (Ref. 171-V23120)</td>
<td>ND^c</td>
</tr>
<tr>
<td>p-AKT</td>
<td>Ser473 (Ref. 171-V21075)</td>
<td>Ser473 Antibody #9271</td>
</tr>
<tr>
<td>p-GSK3β</td>
<td>Ser21/Ser9 (Ref. 171-V23318)</td>
<td>Ser21/Ser9 Antibody #9331</td>
</tr>
<tr>
<td>p-P70S6K</td>
<td>Thr421/Ser424 (Ref. 171-V24155)</td>
<td>ND</td>
</tr>
<tr>
<td>p-MEK1</td>
<td>Ser212/2221 (Ref. 171-V25340)</td>
<td>ND</td>
</tr>
<tr>
<td>p-ERK1/2</td>
<td>Thr202/Tyr204, Thr185/Tyr187 (Ref. 171-V22238)</td>
<td>Thr202/Tyr204 (E10) Antibody #9106</td>
</tr>
<tr>
<td>p-P38MAPK</td>
<td>Thr180/Tyr182, Thr180/Tyr182 (Ref. 171-V21336)</td>
<td>Thr180/Tyr182 Antibody #9211</td>
</tr>
</tbody>
</table>

^b See www.cellsignal.com.
^c ND, not determined.

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VALIDATION PROCEDURE

Validation of the BPA was performed according to the US Food and Drug Administration guidelines (23). Because phosphoprotein reference materials were not available, we were unable to validate some parameters. Therefore, our validation program included assessment of linearity, limit of detection, lower limit of quantification, and upper limit of quantification.

We calculated the limit of detection and lower limit of quantification by using the mean value plus 3 and 10 SDs, respectively, of the fluorescence intensity of 10 blanks containing 250 mg/L BSA. The criterion for acceptance of upper limit of quantification values was based on CV below 20%. Assay linearity was determined from 1/1–1/16 dilutions of the protein extracts from the control cell line.

We fitted the calibration curves by using non-linear 5-parameter logistic equation (Logistic-5PL, Brendman Technologies):

\[ y = \frac{(a - d)}{[1 + (x/c)^b]^m}, \]

where \( x \) is the concentration; \( y \) is the mean fluorescence intensity, \( a \) is the estimated mean fluorescence intensity at zero concentration; \( b \) is the slope of the tangent midpoint; \( c \) is the midpoint; \( d \) is the estimated mean fluorescence intensity at infinite concentration; and \( m \) is the asymmetry parameter.

This regression model allowed better handling of asymmetry, a common cause of poor fit for 4-parameter logistic regression (24), poor replicates, and outliers (e.g., by use of weighting) in the standard curves (25). Goodness of fit for all standard curves was assessed by the method of Davies, which consists of back-calculation of standard value, defined as (observed/expected \( \times 100 \)). Calculated values within 70%–130% of expected values were accepted.

The Pearson correlation coefficient \( r \) was calculated with least-squares linear regression analysis to determine correlation between values measured by BPA and Western blot. Bland–Altman analyses (26) were performed to calculate limits of agreement and systematic errors, and the results were plotted with \( y \) as the value of the difference between BPA and Western blot, \( x \) as the mean of the BPA and Western blot log-transformed values, and the upper and lower limits defined by the mean difference \( \pm 2SD \). In all statistical analyses, the limit of significance was set at \( P < 0.05 \).

A hierarchical cluster analysis was applied to the experimental data obtained with BPA. A euclidian dissimilarity distance and a complete agglomeration method were used. Results were described by a dendrogram in which the \( y \) axis measured distances between clusters. This analysis was performed by the algorithm \( hclust \) implemented into R, a free software environment for statistical computing and graphics.

ASSAY DEVELOPMENT

To assess linearity of BPA, we analyzed successive dilutions of protein extracts from the positive control cell line, within the optimal concentration range recommended (200–900 mg/L). Regression coefficient values \( (r) \) ranged from \(-0.951 \) to \(-1.000 \) \( (P < 0.001) \) (Fig. 1A) for p-EGFR, p-AKT, p-GSK3β, p-P70S6K, p-MEK1, p-ERK1/2, and p-P38MAPK combined as a 6-plex (p-EGFR as a single plex) fitted using the 5-parameter logistic regression algorithm using MCF7 cell dilution. (B), Mean functional sensitivity for multiplex determination, defined as the concentration at which CV reaches 20%.

Results
data lying within the accepted 70%–130% range. We checked for any matrix effect in diluting specimens in protein (BSA) containing buffer to maintain constant total protein concentration at 250 mg/L or 1000 mg/L. No matrix effect was observed because there was no significant variation in either slope or $y$-intercept [See Supplemental Figs. 1 and 2 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue7 for p-AKT (A), p-GSK3β (B), p-P70S6K (C), p-MEK1 (D), p-ERK1/2 (D), and p-P38MAPK (E)].

ASSAY VALIDATION
The established limit of detection, lower limit of quantification, and upper limit of quantification are shown in Table 3. The BPA assay application was validated by use of 49 breast carcinoma surgical specimens obtain from patients before they underwent chemotherapy.

Linear regression was performed to compare BPA with Western blotting as reference method. As a whole, as well as protein by protein, all data obtained from BPA analysis correlated ($P < 0.001$) with data calculated from numerized Western blot with Pearson correlation coefficients ($r$) for each phosphoprotein exceeding 0.70 (p-AKT, 0.85; p-GSK3β, 0.87; p-ERK1/2, 0.83; p-P38MAPK, 0.71) (Fig. 2, left panels).

The Bland–Altman plots of differences and means of results obtained from BPA and numerized Western blot assays (Fig. 2, right panels) showed that for each protein analyzed, $<5\%$ of the values were located outside of the 95% limits-of-agreement interval (p-AKT, 5%; p-GSK3β, 4%; p-ERK1/2, 4%; p-P38MAPK, 2%), demonstrating approximate normality. The systematic differences between the 2 assays were calculated to be 0.337, 0.084, 0.166, and 0.157 arbitrary units (a.u.) for p-AKT, p-GSK3β, p-ERK1/2, and p-P38MAPK, respectively. Interassay and intraassay reproducibility achieved with BPA (Table 3) never exceeded 14.9% and 17.3% respectively, and were within the same range as values usually achieved with numerized Western blot analysis. In 47 of 49 cases, the difference observed between BPA and Western blot values compared to positive control values never exceeded 1 or 2 log (i.e., 10 or 100 a.u., representing approximately 10% of the values measured), respectively, in low (Fig. 2, B and C) and high (Fig. 2, A and D) detection range; and these variations were lower than intra- and interexperimental variations.

ASSAY APPLICATION
The results shown in Fig. 3 demonstrate that large variations (up to several hundred-fold) in phosphoprotein expression could be observed among the tumors.

When the geometric mean was used as the threshold value, overexpression of the PI3K/AKT-related signaling phosphoproteins p-AKT, p-GSK3β, and p-P70S6K were observed in 59%, 53%, and 65% of the patients, respectively. All p-AKT overexpressing samples also overexpressed p-GSK3β, and 86% overexpressed p-P70S6K. In addition, 87% of p-GSK3β–overexpressing samples also overexpressed p-P70S6K.

| Table 2. Goodness of fit for standard curves assed by the method of Davies (see Methods section). |
|----------------------------------------|----------------|--------|
| Phosphoprotein | % of expected values | | |
| Range | Mean | |
| p-EGFR | 94–108 | 99 | |
| p-AKT | 83–125 | 104 | |
| p-GSK3β | 87–121 | 103 | |
| p-P70S6K | 83–116 | 98 | |
| p-MEK1 | 79–130 | 104 | |
| p-ERK1/2 | 94–101 | 98 | |
| p-P38MAPK | 79–130 | 104 | |

<p>| Table 3. Characteristics of the BPA assay in breast carcinoma clinical specimens. |
|----------------------------------------|----------------|----------|----------------|----------------|----------|</p>
<table>
<thead>
<tr>
<th>Phosphoprotein</th>
<th>LOD (MFI)</th>
<th>LLOQ (MFI)</th>
<th>ULOQ (MFI)</th>
<th>Pearson $r$</th>
<th>Interassay CV (%)</th>
<th>Intraassay CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-EGFR</td>
<td>$&lt;110$</td>
<td>$&lt;300$</td>
<td>$&gt;5000$</td>
<td>0.97</td>
<td>10.8</td>
<td>6.8</td>
</tr>
<tr>
<td>p-AKT</td>
<td>74</td>
<td>125</td>
<td>$&gt;25\ 000$</td>
<td>0.99</td>
<td>12.6</td>
<td>8.5</td>
</tr>
<tr>
<td>p-GSK3β</td>
<td>$&lt;20$</td>
<td>$&lt;50$</td>
<td>NDa</td>
<td>0.97</td>
<td>14.9</td>
<td>16.4</td>
</tr>
<tr>
<td>p-P70S6K</td>
<td>27</td>
<td>65</td>
<td>$&gt;25\ 000$</td>
<td>0.98</td>
<td>14.3</td>
<td>3.3</td>
</tr>
<tr>
<td>p-MEK1</td>
<td>$&lt;10$</td>
<td>$&lt;25$</td>
<td>$&gt;25\ 000$</td>
<td>0.99</td>
<td>11.8</td>
<td>5.5</td>
</tr>
<tr>
<td>p-ERK1/2</td>
<td>13</td>
<td>36</td>
<td>$&gt;3500$</td>
<td>0.95</td>
<td>10.4</td>
<td>3.4</td>
</tr>
<tr>
<td>p-P38MAPK</td>
<td>$&lt;45$</td>
<td>$&lt;110$</td>
<td>$&gt;750$</td>
<td>0.94</td>
<td>11.4</td>
<td>17.3</td>
</tr>
</tbody>
</table>

a ND, not determined.
Fig. 2. Assay validation.
Linear regression analysis (left panels) and Bland–Altman plots (right panels) for p-AKT (A), p-GSK3β (B), p-ERK1/2 (C), and p-P38MAPK (D). The dashed lines for the left panels represent the lines of identity; a.u., arbitrary units.
Fig. 3. Assay application.
Expression of representative signaling phosphoproteins p-AKT (A) and p-ERK1/2 (B) in ductal infiltrating breast carcinoma in 49 patients. Tumors using BPA and Western blot. (C), Hierarchical cluster analysis applied to the BPA experimental data. Results of this hierarchical cluster analysis were described by a dendrogram in which the y-axis measures euclidian distances between clusters. Typology of this dendrogram is consistent with the functional representation of EGFR downstream signaling pathway and therefore illustrates biological relevance of the BPA data; a.u., arbitrary units.
p-AKT expression correlated with p-GSK3β expression \( (r = 0.47, P < 0.001) \) and p-P70S6K expression \( (r = 0.53, P < 0.001) \).

All overexpression values ranged between 1.1- and 30.6-fold the threshold value, with median values at 8.1-, 4.9-, and 4.6-fold the threshold value, respectively, for p-AKT, p-GSK3β, and p-P70S6K.

Overexpression of the MAPK-related signaling phosphoproteins p-ERK1/2 and p-P38MAPK was observed in 57% and 49% of the samples, respectively. Most (75%) p-ERK1/2-overexpressing samples also overexpressed p-P38MAPK. All overexpression values ranged between 1.1- and 31-fold the threshold value, with median values at 4.7- and 2.9-fold the threshold value, respectively, for p-ERK1/2 and p-P38MAPK.

p-ERK1/2 expression correlated with p-P38MAPK expression \( (r = 0.39, P < 0.001) \).

Results of the hierarchical cluster analysis are described by the dendrogram in Fig. 3C. Three main clusters of proteins were determined: p-EGFR; p-ERK1/2 and p-MEK1; and p-AKT, p-GSK3β, and p-P70S6K. Typology of this dendrogram is consistent with the functional representation of the EGFR downstream-signaling pathway and therefore illustrates biological relevance of the BPA data.

Although a high level of coexpression was observed (mean 77%, range 67%–82%) within each signaling pathway (PI3K/AKT and RAS/RAF/MAPK), a lower coexpression frequency was found between p-EGFR and the expression of any downstream-signaling phosphoprotein (mean 61%, range 55%–65%). This result illustrates that p-EGFR expression cannot be used as a unique marker for the functionality of all downstream signaling as a whole.

Discussion

Prescription of monoclonal antibodies or tyrosine kinases inhibitors as treatment directed against HER is based on the identification of the targets (HER) in tumor biopsies, mostly achieved using immunohistochemistry or in situ hybridization.

In approximately half of patients with HER2-overexpressing breast cancers, the disease does not initially respond to trastuzumab or it becomes resistant to this drug, an effect attributed to the activation of HER downstream signaling pathways, the PI3K/AKT and RAS/RAF/MAPK pathways (11, 17).

Because of the high risk of resistant disease, many studies have been designed to identify signaling intermediates that could predict the clinical response to anti-HER drugs and be used as diagnostic markers to allow better selection of patients likely to respond. Most studies investigated one or a small set of markers in tumor tissue by using immunohistochemistry or tissue microarray analyses. No global multiple determination of signaling phosphoprotein able to fingerprint HER downstream signaling has been reported, however, probably because of the complexity of the signaling pathways and the limited availability of tumor tissue taken from diagnostic biopsies.

Our study is the first validation and application of a multiplex bead immunoassay that uses BPA assay for the determination of multiple phosphoprotein expression in breast cancer clinical specimens.

A previous reported study investigated various protocols for validation of multiplex beads immunoassays (21) for multiple cytokine determination in clinical specimens. In the present study, we followed the minimal criteria outlined in the US Food and Drug Administration guidance document for industry and bioanalytical method validation (23). This procedure had to be amended, however, because analysis of analyte-free specimen (biopsy) was not achievable, and phosphoprotein standards were unavailable. Under these conditions, the results we achieved using BPA fulfilled all requirements and were fully consistent with those achieved using Western blots with similar protocols and antibodies.

Technically, this study demonstrates some major advantages of BPA for multiple signaling phosphoprotein analysis. When compared with conventional Western blot analyses, on the basis of the determination of 7 proteins in triplicate, the suspension beads protein array requires 5-fold less time than Western blotting, reduces the risk of multiple pipetting errors and reduces hands-on time and therefore cost. Additionally, the use of BPA improves the quality of results because the need for freezing/thawing, typically required for the measurement of multiple analytes by Western blot, is reduced. In addition, we observed that BPA can be used with size-limited tumor specimens (15 mg of tissue) approximately corresponding to a 18-G needle biopsy, a feature that is of particular importance for clinical diagnosis. Compared with immunohistochemistry, the main advantage of BPA appears to be the ability to automate the whole process, giving access to multiple quantitative results. However, BPA does not provide subcellular localization data and does not take into account the heterogeneity of the tumor tissue, as opposed to immunohistochemistry, which allows specific analysis of identified tumor areas.

Activated HER Downstream Signaling Pathways in Breast Tumor Tissue

Upregulation of AKT signaling cascades has been commonly found in a variety of human cancers, including breast carcinoma. In the present study, the constitutive expression of p-AKT observed in 59% of patients agrees with other studies that used immunohistochemistry analysis, reporting p-AKT expression rate from 33% to 70% (27–31) in invasive breast cancers. A lower
rate of p-AKT expression (15%) was recently reported in early stage breast cancers (32), tending to confirm that increased expression of p-AKT could be increased in advanced and metastatic disease. In most of these studies, high expression of p-AKT was also correlated with poor prognosis and resistance to chemotherapy and hormonal therapy. Downstream from AKT, a high frequency (50%) of expression of p-P70S6K and p-GSK3β was observed in the present series, confirming the data previously reported using tissue microarray, which showed 40% of p-P70S6K expression in breast cancer (31). The results achieved in the present study are consistent with the hypothesis that complete constitutive activation of the PI3K/AKT pathway was achieved, because expression of p-AKT was always associated with expression of p-GSK3β and p-P70S6K, as already suggested (33) by tissue microarray data showing that 70% of invasive breast carcinomas expressed p-PDK-1, p-AKT, p-P70S6K, and p-EGFR. The hierarchical cluster analysis illustrates biological relevance of the BPA data, whereas low coexpression frequency between p-EGFR and the expression of any downstream signaling phosphoprotein further suggest that p-EGFR expression cannot be used as a unique marker for the functionality downstream signaling as a whole.

Few data are available regarding the expression rate of phosphorylated-MAPK in breast cancer, and p-ERK1/2 and p-P38MAPK have been mostly associated with poor prognosis and chemoresistance. In this study, the expression of p-ERK1/2 was detected in 57% of the tumors, consistent with immunohistochemistry data reporting expression of p-ERK1/2 in 35%–70% of the tumors (32, 33).

p-P38MAPK was expressed in 49% of the tumors, in agreement with immunohistochemistry data (20%–50%) in breast cancer primary tumors and effusions (34).

As a whole, these data clearly demonstrate the importance of molecular fingerprinting of HER downstream signaling functionality in breast cancer. Monoparametric analyses are not sufficient for individualization of therapeutic regimens, as already shown in colorectal cancer, in which not only the RAS/RAF/MAPK but also the PI3K/AKT pathway has been recently reported (35) to impair clinical response to cetuximab.

In conclusion, this study demonstrated advantages of multiplex HER downstream-signaling phosphoprotein analysis using BPA. This technique is accurate and precise, reduces sample size and time–cost, and provides quantitative results that are fully consistent with Western blot.

Clinically, BPA assays revealed that before treatment initiation HER downstream-signaling functionality can dramatically differ from patient to patient, results that warrant further prospective evaluation of this parameter as a predictive and/or surrogate marker for clinical response to anti-HER targeted therapy. Use of BPA as a prospective screening technique could help identify biomarkers that could be used at diagnosis to help physicians select the most promising individual HER-targeted therapy for cancer patients.

Author Contributions: All authors contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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