SHORT COMMUNICATION

Detection of Truncated HER2 Forms in Formalin-Fixed, Paraffin-Embedded Breast Cancer Tissue Captures Heterogeneity and Is Not Affected by HER2-Targeted Therapy

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Truncated forms of HER2, previously identified in subsets of HER2-positive breast cancer, originate from proteolytic extracellular domain (ECD) cleavage or alternative translation initiation. They lack ECD but may retain intracellular domain functionality, potentially associated with unfavorable prognosis, metastasis, and decreased sensitivity to antibody-based HER2-targeted therapy. To study the distribution of truncated HER2 in breast cancer, we detected loss of membrane-bound ECD independently of its molecular origin in paraffin sections, combining multispectral unmixing of chromogenic duplex IHC for HER2 ECD and intracellular domain with advanced image analysis. HER2 C-terminal fragment 611—transfected MCF7 and 4-aminophenylmercuric acetate—treated SKBR3 cell lines were used as controls. Applying a prototype work flow to whole sections, paired surgical resection/core needle biopsy samples, and paired samples from 69 patients of a phase 2 neoadjuvant clinical trial, we observed unexpected heterogeneity of ECD loss at the single-cell level, and in different areas of individual tumors, indicating that extent and localization of HER2 ECD loss add relevant information to averaging truncated HER2 across whole sections. We show acceptable run-to-run variation (coefficient of variation, <0.15), image analysis results in moderate agreement with conventional slide assessment (Cohen's $\kappa = 0.59$), and no obvious interference with previous HER2-ECD—targeted therapy. We conclude that duplex IHC and digital image processing extend current approaches of truncated HER2 detection. (*Am J Pathol* 2013, 183: 336–343; <http://dx.doi.org/10.1016/j.ajpath.2013.04.010>)

Truncated forms of HER2/neu with oncogenic constitutive kinase activity can be generated by different molecular processes, such as shedding of the extracellular domain (ECD) by proteolytic enzymes,¹ or alternative initiation of translation.² Regardless of their origin, these HER2 forms share the lack of the ECD but have different size and functionality of the remaining protein domains. Various forms generated by aberrant translation contribute to the malignant phenotype of breast cancer cells *in vitro* and promote aggressive biological behavior and metastasis in mouse models.³ The C-terminal fragment (CTF) 611, previously detected in human breast cancer patient samples,⁴ is

associated with down-regulation of the estrogen receptor in HER2-positive breast cancer and may increase resistance to anti-hormonal treatment.⁵ The hypothesis that presence of CTF-611 may confer resistance to HER2-targeted antibody-based therapy^{6,7} conflicts with results showing that CTF-611 detection is associated with good response to trastuzumab,⁸

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indicating that the biological role of truncated HER2 generated by alternative translation initiation remains to be fully clarified. The clinical relevance of truncated HER2 caused by proteolytic ECD shedding is also understood incompletely. The detection of shed HER2 in the peripheral blood has been shown to be prognostic in certain subsets of HER2-positive breast cancer; however, this is not predictive for the response to trastuzumab or lapatinib therapy.^{9,10} Recent findings suggest that changes of shed HER2 after neoadjuvant treatment may have value as a pharmacodynamic marker.¹¹ Immunohistochemistry (IHC) for detection of the HER2 fragment remaining after ECD shedding is not available, probably because of the difficulties of generating specific antibodies binding to the multiple neoepitopes generated by proteolytic cleavage. Similarly, detection of CTF-611 in the morphological tissue context is limited by lack of commercially available and sufficiently validated antibodies suited for IHC. Methods using homogenized tumor samples, such as Western blot (WB) analysis,³ or supernatants of reactions on a tissue section⁷ have the advantage of a quantitative readout, yet lack the possibility to assess heterogeneity in the tissue context of ECD loss in breast cancer. We have recently proposed a novel approach for truncated HER2 detection, visualizing local imbalance between IHC signals specific for ECD and intracellular domain (ICD), as detected by chromogenic duplex IHC. We thereby identified areas with low ECD staining in paraffin sections of breast cancer tissue, regardless of whether the molecular origin is related to HER2 shedding or alternative translation initiation.¹² In the present study, we validate and optimize this approach, with the aim to provide a robust method for truncated HER2 detection in paraffin-embedded material. We further assess the characteristics of this assay when applied to different sample types, including whole tissue sections, matched core needle biopsy specimens, and samples from a biomarker study in the context of a clinical phase 2 trial investigating HER2-ECD-targeted therapy.¹³ We demonstrate diagnostic feasibility and potential limitations of the approach in the context of clinical biomarker studies.

Materials and Methods

Case Selection

For pilot experiments, formalin-fixed, paraffin-embedded tissue of 15 invasive breast carcinoma cases, including 12 cases with paired fresh-frozen breast cancer samples, was obtained from commercial tissue bank providers (Indivumed, Hamburg, Germany; Asterand, Royston, UK; and ProteoGenex, Culver City, CA). The sampling procedures, ethical approval, and informed patient consent are compliant with US and European law. Detailed information is available at Indivumed, Asterand, and ProteoGenex. Eight breast cancer core biopsy samples paired with corresponding surgical resection specimens were obtained as previously described,¹⁴ in accordance with the regulations of the local Ethics Committee (Technical University Munich, Munich,

Germany) for research use of anonymized surplus diagnostic materials. A total of 138 paired core needle biopsy or surgical resection specimens before and after neoadjuvant treatment with trastuzumab and pertuzumab were investigated as part of the biomarker program of the NeoSphere clinical phase 2 trial; patient informed consent and ethics approvals are described elsewhere.¹³

Cell Lines

SKBR3 cells obtained from ATCC (Manassas, VA) were maintained at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. Protease activation by 4-aminophenylmercuric acetate (APMA) was performed to model proteolytic shedding of HER2 ECD, and controls were incubated with corresponding concentrations of dimethyl sulfoxide. CTF-611 was expressed in the HER2-negative cell lines MCF7 and HEK293 (data not shown; both cell lines obtained from the German Collection of Microorganisms and Cell Cultures, Brunswick, Germany) by transfection with a CTF-611 expression vector [kindly provided by Maurizio Scaltriti (Massachusetts General Hospital Cancer Center, Harvard Medical School, Charlestown, MA)], based on pIRES-hyg1 (Clontech, Oxford, UK) using the nonliposomal transfection reagent FUGENE (Roche Diagnostics, Mannheim, Germany). Controls were transfected with empty vector. Cells were harvested mechanically using a cell scraper. Formalin-fixed cells were immobilized in low-melt agarose and embedded in paraffin, following standard protocols.

WB Analysis

Equivalent amounts of protein (15 µg) were separated by SDS-PAGE and analyzed by standard WB techniques using the NuPAGE system (Invitrogen, Frankfurt, Germany), with primary antibodies to HER2 (clone CB11; Novocastra, Wetzlar, Germany) and β-actin (Cell Signaling Technology, Danvers, MA). Visualization was performed using Lumi-Light Plus WB chemiluminescence reagents (Roche Diagnostics) and fluorescent labeling with standard secondary antibodies (Invitrogen). Quantification was performed with the Li-Cor Odyssey instrument (Li-Cor, Lincoln, NE).

IHC Data

Duplex IHC was performed combining two primary antibodies: HER2 ICD was detected using the monoclonal rabbit antibody clone 4B5 (Ventana Medical Systems, Tucson, AZ), followed by a rabbit-specific streptavidin-biotin-based detection reagent (Zytomed, Berlin, Germany) and visualized by Permanent Red (Zytomed). HER2 ECD was detected using a mouse monoclonal antibody raised against HER2 ECD (clone M-15E4F2), followed by a mouse-specific polymer-based detection reagent (ImmPRESS; Vector Laboratories, Burlingame, CA), and visualization by 3,3'-diaminobenzidine tetrahydrochloride (Dako, Hamburg, Germany). Cross-reactivity of M-15E4F2

with other members of the HER family and their phosphorylated forms was excluded by WB, enzyme-linked immunosorbent assay (ELISA), and Biacore-based analysis. Interference of M-15E4F2 with previous trastuzumab or pertuzumab treatment was excluded by ELISA and Elecsys-based competition experiments (data not shown). Antigen retrieval, blocking procedures, washing steps, hemalaun counterstain, and mounting were performed following standard protocols. Negative controls were performed omitting one or both primary antibodies. Positive controls included previously characterized human breast cancer tissue, APMA-treated (APMA⁺) and APMA-untreated (APMA⁻) SKBR3 cell pellets, and a mixture of SKBR3 cells expressing full-length HER2 and CTF-611—transfected MCF-7 cells. Spectral libraries were generated on slides stained with 3,3-diaminobenzidine tetrahydrochloride, Permanent Red, or hemalaun only. During protocol establishment, corresponding sections were stained using different combinations of primary antibodies to ECD and ICD from different species, including the combination of CB11 (Novocastra Science/Leica Biosystems, Nussloch, Germany) and SP3 (Spring Bioscience, Pleasanton, CA), to confirm accuracy of staining. The rationale for choosing a chromogenic approach was the requirement to preserve the full histological context, enabling both visual and computer-aided analysis. Further advantages in the exploratory work flow setting included easier storage and shipment of slides between laboratories. Source Bioscience Laboratories (Nottingham, UK) performed large-scale IHC staining of biomarker samples from the clinical trial, following the in-house developed protocol in batches of 25 slides, plus corresponding controls. Image acquisition and analysis was performed at the Roche Experimental Pathology laboratories.

Image Acquisition

Three to five fields of view (FOVs) per slide were acquired using the Nuance FX system (CRi/PerkinElmer, Hopkinton, MA), following the manufacturer's instructions, in combination with a Leica DM6000 microscope (Leica Microsystems, Wetzlar, Germany), using the 20× objective. For robustness testing, up to 21 FOVs were acquired from the same whole slide. Spectral unmixing was performed after establishing spectral libraries based on single 3,3-diaminobenzidine tetrahydrochloride—, Permanent Red—, or hemalaun-stained control sections for each experimental run in pilot experiments, and for every 100 slides (four batches of 25 slides) in the large-scale staining of clinical samples.

Image Analysis

The modular image analysis work flow consisted of three parts: i) a preprocessing step for tissue segmentation and classification and identification of individual cells and their subcellular compartments (nucleus, membrane, and cytoplasm), ii) a manual interaction step to control accuracy of the detection of viable invasive tumor areas, and iii) the

generation of multiple output parameters, including the ratio of ECD/ICD staining signals of cell membranes of intact, morphologically complete tumor cells and the ratio of membrane/intracellular ICD staining (Definiens Developer XD; Definiens, Munich). Normalization, output, and graphical display of data were performed using R, version 2.13.1 (<http://www.r-project.org>), or Definiens Image Miner software version 2.0.2 (Definiens).

Normalization, Data Exploration, and Statistical Considerations

ECD/ICD measurements of single cells that exceeded the normally observed range of values were excluded from the analysis. These outliers were identified for each individual FOV and were defined as being >1.5 interquartile ranges (IQRs) higher than the third quartile of the single-cell ECD/ICD values. For each FOV, the overall mean of the ECD/ICD measurements of the remaining cells was taken as an output parameter. Each case was summarized by averaging its corresponding FOVs. The upper range of evaluable ECD/ICD measurements and the exclusion of single cases were defined using the reference tissue (control case 2, $n = 14$). Again, outliers were defined as being >1.5 IQRs higher than the third quartile of the ECD/ICD values of the reference case. During the pilot phase, normalization of each experiment was performed in reference to untreated (APMA⁻) SKBR3 cells processed in the same experimental run. To scale up the analysis to large sample numbers, normalization was performed in reference to the median of all APMA⁻ cell images. In addition, results obtained from untreated SKBR3 cells were used to define the range of breast cancer cases with high, intermediate, and low ECD/ICD ratios. The lowest observed ECD/ICD value for APMA⁻ cells was used as the lower bound for ECD/ICD high. Statistically, values in the range [quartile 25 (Q25) – $(1.5 \times \text{IQR})$; quartile 75 + $(1.5 \times \text{IQR})$] could result from APMA⁻ cells. Thus, $Q25 - (1.5 \times \text{IQR})$ was used as the lower bound for ECD/ICD intermediate. All ECD/ICD values lower than $Q25 - (1.5 \times \text{IQR})$ were considered ECD/ICD low. APMA-treated (APMA⁺) cells were used to confirm the choice of these thresholds. The coefficient of variation (CV) was used to compute the relative variation of the ECD/ICD ratio across runs, with $\text{CV} = \text{SD}/\text{mean}$. Robustness of the ECD/ICD values was determined by calculating the sampling variation using a bootstrapping approach (2500 repetitions) implemented in R version 2.13.1 (<http://www.r-project.org>), either for the number of FOVs or the number of cells. Data are displayed as CV of the 2500 means per fixed number of cells or FOVs, respectively. Agreement between the ECD/ICD ratio generated by image analysis and the pathologist's (F.F.) assessment for loss of ECD (positive, intermediate, or negative) was visualized using box plots. Cohen's linearly weighted κ statistic¹⁵ was used to rate the agreement between computer-based image analysis and the pathologist's evaluation. For this, ECD/ICD ratios were categorized into ECD/ICD high, intermediate, and low using the previously introduced, preliminary thresholds.

Results

Feasibility of Truncated HER2 Detection in Formalin-Fixed, Paraffin-Embedded Cells and Tissues by Chromogenic Duplex IHC

We optimized the work flow composed of duplex IHC and image analysis¹² (Supplemental Figure S1) to the range of variation observed in a pilot series of commercially acquired breast cancer samples of controlled pre-analytical processing quality (Figure 1A), and confirmed that the results reflected loss of ECD based on the major molecular mechanisms. Proteolytic cleavage of the ECD after treatment of HER2-overexpressing SKBR3 cells with the protease activator, APMA, resulted in a visually detectable decrease of the brown ECD signal. The HER2-negative breast cancer cell line, MCF-7, transfected to express CTF-611, showed exclusively red staining (ICD signal), confirming accurate detection of truncated HER2 generated by alternative translation initiation (Figure 1B). Compared with formalin-fixed cell-line pellets, breast cancer tissues showed increased heterogeneity of IHC staining (Figure 1, D and E), including coherent areas of variable color (Figure 1C), single cells within solid tumor areas (Figure 1A), and, in some cases, subtle differences between tumor center and invasive edge (Figure 2A). Visual comparison between fluorescent staining and chromogenic IHC showed accuracy of ECD/ICD detection (Supplemental Figure S2A).

Truncated HER2 Detection in Breast Cancer Whole Slides versus Core Needle Biopsy Specimens

Because the use of biomarker assays in clinical studies in breast cancer requires feasibility in core needle biopsy samples, we excluded staining variation due to differences in processing of needle biopsy specimens versus surgical resection specimens (eg, cold ischemia time and tissue fixation) using material from a previous study on pre-analytical processing in IHC.¹⁴ Paired samples ($n = 8$; including three cases with an HER2 score of 3+) were studied. The staining result confirmed that representative core needle biopsy specimens of 8- to 10-mm length and sufficient tumor content can be expected to capture relevant structures in small tumors (Figure 2, A–E). The shorter cold ischemia time and faster fixation of small needle biopsy specimens were associated with slightly enhanced staining intensity, without obvious effects on the staining color (Figure 2, C and D).

Comparison of Image Analysis and WB Analysis in Truncated HER2 Detection

A pilot series of 12 cases with paired formalin-fixed, paraffin-embedded tissue/fresh-frozen tumor samples was investigated to compare truncated HER2 detection by IHC (Figure 3A) with WB analysis (Figure 3B). By using

Figure 1 Truncated HER2 detection. **A** and **B**: Controls confirming accuracy of truncated HER2 detection in representative human breast cancer cases and cell lines. **A**, left panel: Breast cancer case expressing a predominantly full-length receptor showing evidence for decreased ECD/ICD ratio only in single cells, and breast cancer case with decreased ECD staining (right panel), indicating presence of truncated HER2. Brown membrane staining suggests the presence of a full-length receptor in single cells (asterisk). **B**, left panel: SKBR3 cells (untreated control) predominantly expressing full-length HER2. Right panel: APMA-treated SKBR3 showing loss of the brown ECD staining. Middle panel: Mixture of MCF7 cells after stable transfection with an expression vector containing CTF-611 (asterisk) in a background of untreated SKBR3 cells. **C–E**: Results of the digital image analysis. **C**: Representative images for quality control, allowing the pathologist to correlate visual assessment with the color-coded digital measurements. **D**: Graphic display of mean ECD/ICD ratio averaged across multiple FOVs; individual measurements (black squares) and mean values (red asterisks) are given. **E**: Stacked bar graphs indicating the percentage of high, intermediate, and low ECD/ICD ratios across individual cases.

fluorescent detection and the Li-Cor Odyssey system for quantification, we determined the intensity of bands at 95 and 185 kDa (truncated p95HER2 and full-length p185HER2). A plot of the ratio of p185HER2/p95HER2 against the mean ECD/ICD signal ratio obtained by image analysis confirmed a general trend consistent with the notion of increased p95HER2 being associated with a lower ECD/ICD ratio (correlation coefficient $r = 0.72$) (Figure 3B). Comparison with the scatter plot of the individual image analysis results per FOV in each case (Figure 3A) suggested

Figure 2 Surgical resection and core needle biopsy comparisons. **A–E:** Comparison of surgical resection material with core needle biopsy specimens. **A:** Full cross section of tumor after surgical resection. Note the subtle heterogeneity, with a slight trend toward more prominent red staining at the invasive edges of the tumor regardless of whether the tumor border was embedded adjacent preexisting breast tissue (area corresponding to **B**) or immediately exposed to fixative (area corresponding to **C**). **B:** Enlarged view of area B of the surgical resection. **C:** Enlarged view of area C of the surgical resection; the rectangles in [Figure 1A](#) only approximately match the small areas shown at high resolution. **D:** Corresponding core needle biopsy specimens of the same tumor showing largely identical staining, possibly with a slightly more intense overall staining signal. **E:** Enlarged view of the area **E** of the core needle biopsy specimen.

ECD/ICD ratio variation that was not detected in the WB-based results averaged per case.

Confirming Feasibility and Robustness of Image Analysis in a Larger Series of Samples

To finally run the prototype work flow at the scale of a clinical trial, a series of 26 experimental runs for breast cancer tissues was performed to ensure robustness of the staining and analysis procedure across multiple experiments ([Supplemental Figure S2B](#)). Seven of these runs included APMA^{+/−} SKBR3 cell pellets. Run-to-run variation was within a narrow range for cell lines (CV = 0.02 for APMA-treated SKBR3 cells, and CV = 0.11 for untreated cells), and showed greater, but still acceptable, variation in breast cancer control tissues (CV = 0.14 for

case 2 with predominantly truncated HER, and CV = 0.15 for case 4 with predominantly full-length HER2). This observation reflected the greater heterogeneity in cancer tissue if compared with cell-line pellets. Comparing automated image analysis with the manual assessment by a pathologist, a moderate concordance was observed in the series of 138 paired samples obtained from 69 patients (Cohen's κ = 0.59). Of 138 samples, 91 (66%) were in agreement. Discordant results between manual and automated analyses were mainly observed for assignment of the intermediate and ECD loss group, with a trend toward more cases assigned to the loss of ECD group by visual assessment (37 of 138) than by automated analysis (25 of 138).

Impact of Tissue Composition and Sample Quality on Image Analysis Robustness

The accuracy of automated image analysis is critically dependent on the quality and composition of the original image data. Given the heterogeneity observed in the pilot series of archival samples, we addressed robustness of the approach by determining how many FOVs representative for the viable invasive tumor component are needed to minimize variation. We found that image analysis results reached a stable range of variation after three to eight FOVs at $\times 20$ magnification, or 250 viable, intact cells, depending on tumor cell size, tumor composition, and percentage of viable tumor regions within individual FOVs ([Supplemental Figure S3](#)).

Figure 3 Pilot series of breast cancer cases illustrating the range of ECD/ICD variation across different tumors and the heterogeneity of truncated HER2 within individual samples. **A:** Graphical display of mean ECD/ICD ratio (red asterisks), after normalization on the mean ECD/ICD ratio of non-APMA-treated SKBR3 cells. **B, left panel:** ECD/ICD ratio plotted against WB results (ratio of the 185- and 95-kDa band intensity) confirming a general trend toward consistent results with both approaches (correlation coefficient r = 0.72). **Right panel:** Representative WB of the cases of the pilot series showing full-length HER2 and its truncated forms, including p95. β -Actin serves as loading control, and SKBR3+APMA serves as positive control for p185 and p95.

Truncated HER2 Detection at the Scale of a Clinical Trial: Impact of HER2-ECD–Targeted Therapy and Preliminary Definition of Potential Thresholds for Defining ECD Loss

To demonstrate that previous HER2-targeted therapies using therapeutic antibodies to ECD epitopes (trastuzumab and pertuzumab) do not interfere with binding of the diagnostic primary antibody, we analyzed samples from a subset of patients in the NeoSphere study.¹³ Selected samples fulfilled the following criteria: i) no complete pathological response, ii) HER2 score of 3+ at diagnosis, and iii) at least one unstained slide before treatment (BT) and after treatment (OT) available. Independent of the study arm (group A, trastuzumab plus docetaxel; group B, pertuzumab and trastuzumab plus docetaxel; group C, pertuzumab and trastuzumab; and group D, pertuzumab plus docetaxel), we found any type of change between pretreatment and post-treatment ECD/ICD ratio, including increase (43%) and decrease (57%) (Figure 4A). A statistically significant difference in ECD/ICD ratio change between the study groups was not observed (Figure 4B). We further used the sample collection

to propose a preliminary quantitative definition of ECD loss, as determined by IHC. This was done by using a rather conservative estimate for the lower boundary of the definition of full-length receptor (the lowest observed ECD/ICD ratio in non-treated SKBR3 cells) (Figure 4, C and D), and by calculating the higher boundary of the ECD low category based on quantile-based outlier analysis. Based on this preliminary definition, of 138 paired samples from 69 patients, 18% (25/138) were classified as low ECD/ICD ratio, 53% (73/138) were assigned to full-length receptor/high ECD/ICD ratio, and 29% (40/138) could not be categorized because of an intermediate global ECD/ICD ratio. Comparing the ECD/ICD ratio obtained by image analysis with the manual assessment, there was a trend toward applying a slightly higher threshold for the visual analysis by a pathologist (approximately 0.85) than the calculated cutoff for automated assessment (0.79) (Figure 4E).

Discussion

The present study confirms the feasibility of chromogenic duplex IHC to detect truncated HER2 in paraffin-embedded

Figure 4 Antibody-based HER2-targeted therapy does not affect the detection of HER2 ECD/ICD ratio. Analysis of a subset of study samples from all four treatment arms for which baseline and post-treatment biopsy specimens were available ($N = 69$ patients). **A:** Matched patient baseline (BT) and OT samples are shown in a single column (x axis patient ID, grouped by treatment arm). ECD/ICD ratios observed for BT and OT samples do not differ between the different treatment groups. Ctrl, control. **B:** Summary of the results presented in **A** by calculating the ECD/ICD ratio difference between the OT and BT for each treatment arm and plotting the results summarized as box plots. No difference between the treatment arms is observed. **C:** Summary of the values for APMA-treated and APMA-untreated SKBR3 presented in **A**. Thresholds for ECD/ICD ratio high, intermediate (int), and low are calculated from the sample distribution of untreated SKBR3 cells (as described in *Materials and Methods*). **D:** Matched patient BT and OT samples are shown in a single column, ordered according to the ECD/ICD ratio. Thresholds calculated in **C** and APMA-treated and APMA-untreated cells are displayed as a reference. **E:** Comparison of image analysis and pathologist's assessment reveals moderate agreement. Box plot comparing the pathologist's categories with image analysis ECD/ICD ratios shows a general agreement, but a higher threshold, of the pathologist for loss of ECD [positive (pos) box plot has a median of 0.8; quartiles 25, 75 = 0.75, 0.85, whereas image analysis ECD/ICD ratios <0.79 were scored as ECD/ICD low]. Green circles, APMA-treated SKBR3 cells (positive controls); red circles, untreated SKBR3 cells [negative (neg) controls]; blue circles, baseline breast cancer biopsy species before treatment; yellow circles, post-treatment biopsy specimens of residual tumors.

breast cancer samples. To our best knowledge, this is the first time that advanced image analysis analyzing concomitantly stained ECD and ICD of a membrane-bound receptor has been used for exploratory biomarker analyses in a phase 2 clinical trial to investigate the impact and relevance of truncated HER2 receptors in the context of HER2-targeted therapies. Given that published results on the predictive value of CTF-611 expression were not clearly consistent,^{7,8} a major achievement of this approach is to enable detection of truncated HER2 independently of its molecular origin (eg, alternative translation initiation or proteolytic cleavage, which has not been possible with available methods).^{9,10} In view of the increasing need to advance biomarker analyses in large neoadjuvant clinical studies,^{8,13,16} we anticipate that further development of the assay principle and future applications in clinically annotated samples will contribute to a deeper understanding of the biological role of truncated HER2 molecules. We observed unexpected variation of staining signals across individual samples, suggesting previously unknown heterogeneity of the distribution of truncated HER2 forms. The genomic amplification of the *HER2* locus is generally thought to be more homogeneous in breast cancer than in many other types of cancer.¹⁷ Likewise, the expression of truncated HER2 as the result of alternative translation initiation has been thought to be a genuine homogeneous feature of individual tumors throughout all tumor cell populations,^{4–6} rather than a focal, or functionally regulated, mechanism in specific tumor areas. We studied a cohort of patients with breast cancer whose HER2 IHC 3+ scoring tumors were supposed to be homogeneously driven by constitutive HER2 protein overexpression because of *HER2* gene amplification as the underlying genomic aberration. We were, however, aware that, in rare cases, intrinsic genomic heterogeneity may be also present in breast cancer.^{18,19} Because sequential tissue slides of the study cases were not available for fluorescence *in situ* hybridization, and fluorescence *in situ* hybridization data were not reported for all HER2 score 3+ cases, we were not able to fully exclude genomic tumor heterogeneity. Despite this limitation, the observed heterogeneity of ECD loss may be explained by proteolytic shedding of HER2 as a contributing, if not the prevailing, mechanism for production of the truncated HER2. Heterogeneous staining signals may reflect local differences in the expression of matrix metalloproteinases, either because of intrinsic tumor heterogeneity or in response to interaction with the adjacent inflammatory tumor microenvironment. The analytical approach we present herein provides the technical prerequisites to comprehensively address the spatial dimension of ECD loss. As the image analysis work flow captures tumor cells as individual image objects, it leverages subsequent analyses in which the comprehensive information on local differences in HER2 ECD loss could be linked to other parameters of interest (eg, regional activation of HER2-associated oncogenic signaling pathways, such as mitogen-activated protein kinase and phosphatidylinositol

3-kinase/AKT,¹⁴ or tumor-infiltrating immune cells).²⁰ For this type of analysis, we propose to further explore the advantages of using conventional bright-field microscopy for image acquisition, capturing morphological categories, such as tumor center, stroma and immune cell interaction, or invasive tumor edge, for further analysis.

The observed heterogeneity raises important concerns about the possibility of sampling errors and potential impact of pre-analytical tissue processing. By using high-quality samples from certified commercial tissue providers and large academic medical centers, which comply with stringent standard operating procedures, we have minimized the risk of preprocessing artifacts. However, signal changes due to ischemia and fixation can never be completely excluded.¹⁴ Moreover, it has recently been shown that antibody binding to HER2 ECD epitopes can be altered in unexpected ways by proteolytic cleavage,²¹ a potential risk for assay variation that we addressed by appropriate cell-line controls. Regarding potential sampling errors, we could show that our analysis approach, based on individually acquired FOVs, is sufficiently robust for exploration of ECD loss in an exploratory setting and that the use of core needle biopsy specimens can appropriately represent the whole tumor, provided optimal tumor and biopsy size and sample quality. The limitations of this prototype work flow, including its limited dynamic range, the manually performed IHC assay, and the still preliminary thresholds to define areas of low ECD/ICD ratio, should be addressed in further development of the approach. In general, any approach that uses color deconvolution or multispectral unmixing of chromogenic IHC in exploratory biomarker studies will face certain limitations.²² Still, the method we present herein holds promise to significantly add biologically relevant information not captured by available methods of detecting HER2 ECD loss and should, therefore, be evaluated in larger clinically annotated patient cohorts.

In summary, we showed the feasibility of a novel digital image analysis approach for detecting truncated HER2 and provide data indicating that it can be applied in biomarker programs of a phase 2 clinical trial. We believe that the high potential of combining multispectral analysis with object-oriented image analysis will contribute to further insights into the previously unrecognized heterogeneity of truncated HER2 in breast cancer, and that further technical development will overcome limitations of the exploratory prototype work flow. We expect that the feasibility of this approach in paraffin-embedded material will help to clarify the biological features of truncated HER2 forms in a clinical context.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.04.010>.

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